# MICROÖRGANISMS CAUSING FERMENTATION FLAVORS IN CANE SIRUPS, ESPECIALLY BARBADOS "MOLASSES"[1]

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During recent years a considerable quantity of a high grade cane sirup has been shipped into this country from the Island of Barbados, British West Indies. This sirup, commonly known as "Barbados Molasses," is sold at a slightly higher price than domestic sirups and is generally distributed in barrels, a comparatively small quantity being sold in retail packages. Upon receipt in this country, the molasses is stored or "cured" in barrels for some months prior to distribution. During this curing period several reactions take place. First there is an active yeast fermentation accompanied by vigorous gassing, during which most of the alcohol is produced. A distinct odor and flavor of alcohol develop. After the fermentation stage, a rum-like flavor develops, and increases in intensity upon prolonged storage.

Browne (1919) in relating his observations of the sugar and sirup industry of Barbados, Tempany (1913) and Watt and Tempany (1905) describe the methods of making "fancy molasses," which is actually sirup. Cane juice, clarified with milk of lime, is boiled down in open tayches. Factories making this type of product maintain a supply of "sour" juice, i.e., cane juice which has undergone natural alcoholic and then acetic fermentations. During the process of concentration sour cane juice is added to invert sufficient of the sucrose to prevent subsequent crystallization, the excess of the volatic acid being boiled away in the tayches. The sirup is evaporated, with continuous skimming, to

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about 36° Baume hot, or about 42° Baume cold. When cooled this sirup is run into puncheons, hauled to port, and stored in underground cisterns, pending shipment. Thus the output from many small factories is blended, overcoming individual variations.[2] Being free from sulphur dioxide, hydro-sulphites, and an excess of lime, these sirups possess a characteristic taste and aroma.

Allen (1906), discussing the manufacture of Jamaica rum from sugar cane products, pointed to the importance of *Bacillus butyricus* and *Bacillus amylobacter* and allied forms in the production of organic acids which are essential in the flavoring of rum. He also described the part played by yeasts in the production of alcohol, a forerunner of the aromatic esters. During the earlier part of the fermentation of the cane juice, members of the *Saccharomycetes* predominated, but as the acidity developed members of the *Schizosaccharomycetes* became prevalent. At the end of the fermentation only bacteria could be isolated from the "dead" liquor. Allen contends that at the end of the yeast fermentation the bacteria utilize the dead yeast cells as a source of food. *Bacillus mesentericus* was thought to produce butyl alcohol in the fermenting mixture. Minute quantities of higher alcohols, furfural, and aldehydes were present, presumably the products of microörganisms.

## EXAMINATION OF MOLASSES

Since the changes in the flavor of curing molasses suggest microbial activity, an examination was made of the micro-flora of several samples of Barbados molasses. Also, in order to facilitate further the development of methods for the production of rum flavors in domestic sirups a chemical examination was made of the volatile materials of two barrels of cured Barbados molasses.

## ISOLATION AND STUDY OF YEAST

A microscopical examination of sediment (obtained by centrifuging a mixture of equal parts of molasses and water) revealed the presence of numerous yeast and bacterial cells. Several

[2] Information from importer.

attempts to obtain yeast cultures from cured molasses by plating on malt-extract agar and on cane sirup agar[3] were unsuccessful. Finally, tubes of sterile malt-extract broth containing approximately 25 per cent cane sirup were inoculated with the sediment from the molasses. The tubes were incubated at 30°C. for 12 to 18 hours; plates were then poured with cane sirup agar. These plates after six days incubation at 30°C. revealed the presence of a variety of yeast colonies, which were picked and transferred to slants of cane sirup agar, and were later found to be the true agents of the alcoholic fermentation in Barbados molasses.

Eleven cultures were thus obtained from two samples of Barbados molasses made in 1932 and 1933. Cultures from the 1932 molasses were designated by the number 32; those from the 1933 molasses by the number 33. The cultures, maintained on slants of clarified honey agar (Hall and Lothrop, 1934), were studied morphologically, culturally and physiologically. As a result of these studies the yeasts were divided into two groups.

Group I. *Zygosaccharomyces nussbaumeri* Lochhead and Heron. This group includes yeasts numbered 32-1, 32-3, 33-1, 33-5 and 33-6.

The characteristics of this yeast agree essentially with those of *Z. nussbaumeri* Lochhead and Heron (1929). A point of difference is the formation of scum by cultures 32-1, 32-3, and 33-5, but this single difference is not considered sufficient for disagreement with the type species. This yeast was first isolated from honey by Fabian and Quinet (1928) who named it *Z. priorianus* Klocker. Later, however, Lochhead and Heron, discovering an error in Guillermond (1920), renamed it *Z. nussbaumeri.* It has since been isolated from fermented maple sirup by Fabian and Hall (1933).

Group II. *Zygosaccharomyces major* Takahashi and Yukawa. This group includes yeasts numbered 32-2, 32-4, 32-6, 33-2, 33-3 and 33-4.

Since the morphological and cultural characteristics of this

[3] Cane sirup agar was made by adding one part of cane sirup to 2 parts of 2 per cent nutrient agar; the reaction adjusted to pH 6.8 to 7.0 before sterilization.

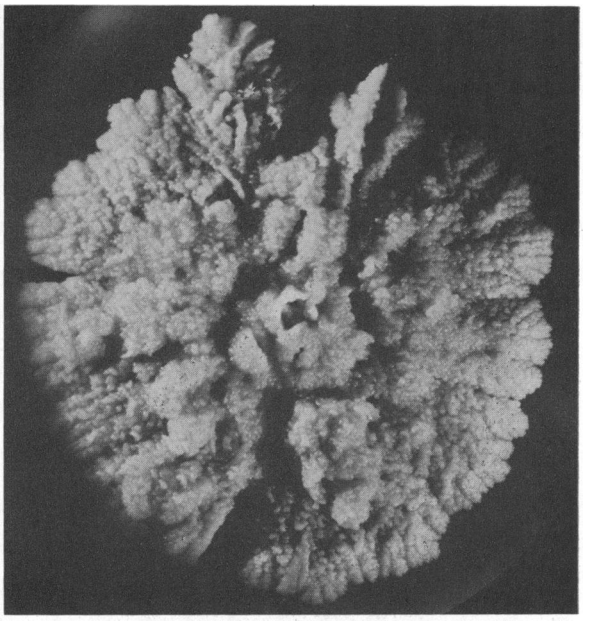

FIG. 1. GIANT COLONY OF YEAST CULTURE 32-1. GROUP I.

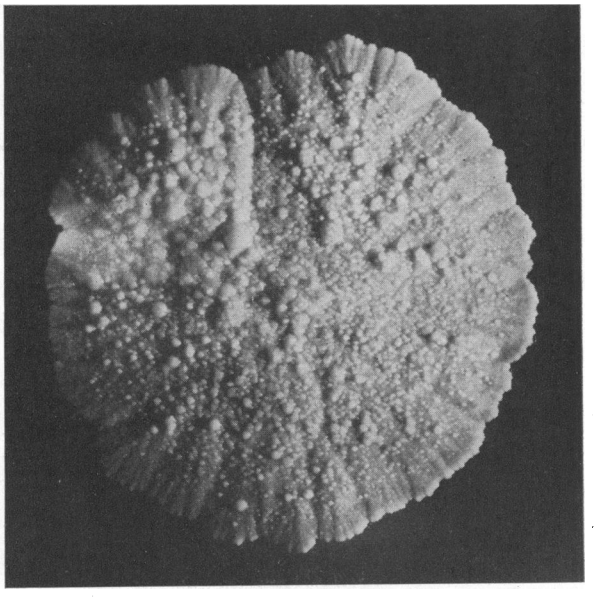

FIG. 2. GIANT COLONY OF YEAST CULTURE 32-4. Group II.

group correspond essentially with those of the yeast isolated by Takahashi and Yukawa from ripening "Shoju" (1912); it is felt that it should be called *Z. major* Takahashi and Yukawa.

The growth in the giant colonies of cultures of Group I (fig. 1) is abundant, and tends towards irregular folds and wrinkles. The edge of the colony is irregular and marked by deep indentations. Gassing frequently occurs within the colony, often causing deep fissures and a crateriform appearance. The growth of giant colonies of cultures of Group II (fig. 2) is moderate, flat and warty. The edge of the colony is irregular, and is free from the warty appearance until after several days of incubation. The color is grayish bordering on brown. The primary growth is dull while the secondary, or warty growth, is glistening.

## ISOLATION AND STUDY OF BACTERIA

A study was made of the bacteria, in Barbados molasses, consistently appearing on solid and in liquid media. Plates, made of each sample, were poured with 2 per cent plain nutrient agar, 1 per cent glucose agar, 25 and 50 per cent sucrose agar, and 25 and 50 per cent cane sirup agar, and incubated under aerobic and anaerobic conditions at 20°, 30°, 37° and 55°C. Meat tubes were inoculated from physiological-saline molasses suspensions which had been heated at 80°C. for 10 minutes. To insure a strictly anaerobic condition the meat tubes were heated for 30 minutes in flowing steam prior to inoculation. The inoculated tubes were sealed with autoclaved petrolatum and incubated at 30° and 37°C.

A variety of colonies developed under practically all the test conditions in agar media. No one type of organism predominated, and subsequently none could be found to play a part in the flavoring of sirups. Mold colonies were frequent on plates incubated at lower temperatures under aerobic conditions. The molds proved to be mostly *Penicillium* and *Mucor*. Vigorous gassing and clouding of the broth in all the meat tubes occurred within 24 to 48 hours, so that anaerobic organisms appeared to be most prevalent. Later these were found to play a part in the flavoring of laboratory samples of sirups.

Pure cultures of anaerobic bacteria were obtained by making a

series of shake cultures in yeast-water-agar, which were incubated for 2 or 3 days at 37°C. and then at 30°C. for several days. Although colony development was often slow many well-isolated colonies developed. Subcultures were made from these colonies into tubes of chopped meat medium. All morphological studies were made from meat tubes. Tubes of carbohydrate broths, etc., were inoculated with organisms obtained from the supernatant fluid from the meat tubes.

The growth in the meat-medium tubes was vigorous, accompanied by gassing and a highly putrefactive odor. There was reddening of the meat with subsequent darkening. An examination of cultures revealed a Gram-positive rod occurring singly, in pairs, and in chains. The average length of the cells was 4.5$\mu$ the shortest was 3.2$\mu$, and the longest was 5.8$\mu$. The width was from 1.0$\mu$ to 1.5$\mu$. No motility was observed. Flagella were not demonstrated. Oval spores were formed and were usually terminal. The rods were not swollen on sporulation.

The organism did not grow on aerobic glucose agar slants but beaded, grayish, viscous colonies appeared under anaerobic conditions. There was no growth on plain agar slants under either condition. In yeast-water-agar stabs a filiform growth appeared after 2 or 3 days along the line of inoculation, followed by fleecy outgrowths extending several millimeters into the medium. Growth occurred in plain broth and was accompanied by a putrefactive odor. Gelatin stabs were completely liquefied in 24 hours at 20°C. Brain medium was blackened. Coagulated albumen was softened. Nitrates were not reduced to nitrites. Indol was produced from tryptophane broth.

Acid and gas were produced from mannose, fructose, glucose, sucrose, maltose and glycerol. Salicin, inulin and galactose were feebly fermented. Plain and litmus milk was completely peptonized after six days incubation at 30°C., gas being produced. This culture is identified as *Clostridium saccharolyticum* Bergey *et al.* Variations from the type species such as motility and spore size were observed. Since all other characteristics correspond so closely with those described for the type culture it does not seem that these differences warrant naming a new species.

When samples of sirup were fermented in the absence of *C. saccharolyticum* the characteristic rum flavor did not develop. That this organism utilizes the residual yeast cells and other organic matter in molasses and produces substances that contribute to the formation of rum flavors seems highly probable.

### CHEMICAL EXAMINATION OF VOLATILE MATERIALS

The volatile constituents were distilled with steam from a barrel of cured Barbados molasses. The distillate was fractionated with a Glinsky column, and 2444 cc. of ethyl alcohol boiling below 80°C. were obtained. This alcohol appeared to carry most of the characteristic Barbados flavor. Careful fractionation of it gave a small amount of highly flavored distillate in which furfural was identified by its semi-carbazone. After the removal of the furfural an aldehyde was obtained by treatment with sodium bisulfite. It had an unmistakable odor of vanillin, but chemical proof of the presence of vanillin was lacking. In the nonaldehyde fraction butyl alcohol was recognized by its odor. The constituents forming the true Barbados flavor were not isolated.

A second barrel of Barbados molasses was later distilled, and 15 gallons of distillate were collected. Acetaldehyde came over in the first distillate and was identified by its semicarbazone. Ethyl alcohol boiling at 78.4 to 79.0°C. (2200 cc.) was also obtained from this distillate. The characteristic rum flavor was obtained on fractionating the alcohol and was also obtained from the watery distillates with ether. Hydrolysis or polymerization of the flavor fraction caused great loss in the essential oil which is responsible for the flavor, and only a very small amount was recovered. This had a powerful rum odor. From the flavor fraction and the last runnings of the alcohol, furfural was isolated as its semicarbazone.

The isolation and identification of chemical compounds, which usually result from the metabolism of yeast and bacteria, add additional support to the belief that the flavoring of Barbados molasses is the result of a fermentation similar to that occurring in the manufacture of rum. The chief organism concerned is

necessarily a yeast, upon which reliance must be placed for the production of alcohol. The volatile esters, higher alcohols, furfural and aldehydes probably result from the metabolism of bacteria.

Further evidence to support the theory of rum fermentation in Barbados molasses has been obtained by the development of fermentation flavors in domestic cane sirups. Cultures of the yeast and bacteria previously described have been propagated in samples of high grade cane sirups and have resulted in the production of flavors not unlike those of Barbados molasses.

## SUMMARY AND CONCLUSIONS

1. A microbiological examination was made of two samples of Barbados molasses.

2. The yeasts *Zygosaccharomyces nussbaumeri* Lochhead and Heron and *Zygosaccharomyces major* Takahashi and Yukawa were isolated and their rôle determined in the flavoring of cane sirups.

3. Of the bacteria *Clostridium saccharolyticum* Bergey *et al.*, was isolated and probably aids in the flavoring of cane sirups.

4. The volatile substances ethyl alcohol, furfural, acetaldehyde, and butyl alcohol were obtained by distillation.

5. The relationship of yeast and bacteria to the production of rum flavors in domestic cane sirups is suggested.

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