STUDIES ON THE FERMENTATION OF TOBACCO [1]

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INTRODUCTION

After curing, cigar-leaf types of tobacco in particular are allowed to undergo one or more fairly definite periods of fermentation or "sweating." This process is characterized chiefly by an exchange of gases, the generation of heat, and a modification of the flavor and aroma of the leaf. The aging process in tobacco is not in all cases clearly distinguishable from fermentation, except that the rate of activity in the latter is more rapid and results in an appreciable liberation and accumulation of heat and gases. Although the subject of fermentation has received considerable attention in the past no satisfactory technic for measuring improvement in quality has been devised, and estimates of the progress of fermentation are dependent largely upon the opinion of those experienced in judging tobaccos.

As there is little exact knowledge concerning the nature of the process of fermentation, comprehensive investigations from several different angles will be necessary to establish the facts. The present investigations, in which the Dewar-flask method was used, are primarily concerned with the possible relationship between micro-organisms and the changes involved in the fermentation of cigar-leaf types of tobacco.

EARLIER STUDIES

The chemical changes occurring in tobacco during fermentation have been given particular attention by several investigators, but need not be reviewed here. It should be recalled, however, that tobacco fermentation is generally believed to be an oxidation process, and the close relation of air to the results secured has been generally recognized. Analyses show significant decreases in nitrogen compounds, including nicotine, and other organic substances, accounting for a loss of solid matter sometimes exceeding 5 percent, during fermentation. The total loss of weight, including that of free water, may be considerably higher during the process. On the other hand there is a marked liberation of ammonia and carbon dioxide gases as a consequence of this activity. The improvement of the aroma, flavor, burn, and other qualities is to be regarded as of major importance even though not chemically determinable. Separating the essential and desirable changes from incidental changes constitutes one of the chief difficulties of the chemical aspects of the problem.

The previous investigations which are of most interest in relation to the results secured in the present investigations are those dealing with the possible relationship of enzymes and micro-organisms to the fermentation process. There have been certain claims (*17*) [2] and

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[2] Reference is made by number (italic) to Literature Cited, p. 159.

there is some evidence for the contention that, given the proper conditions, fermentative changes may occur in the absence of any enzymic or microbial activity, but this theory has not received much support. The problem seems rather to involve the relative importance of the enzymic and the microbial activities during normal fermentation, even though these may be regarded only as agencies speeding up the rate of oxidation and other chemical changes.

As early as 1858 an analogy between tobacco fermentation and alcoholic fermentation was implied by Koller (*10*), who added yeast with the purpose of increasing the rate of tobacco fermentation. Later, tobacco fermentation was compared with that of silage (*4*), "brown" hay (*1*), manures (*19*), etc. The bacterial theory of tobacco fermentation was most definitely brought forward in 1891 by Suchsland (*21*), who isolated bacteria from sweating tobacco, prepared pure cultures, and inoculated these back into tobacco. His claim that the flavor and odor of a specific type of tobacco could be developed in another type through the use of bacterial cultures has not, however, been substantiated. Miciol (*15*), Dávalos (*3*), Vernhout (*22*), Behrens (*1*), Koning (*11*), Jörgensen (*9*), and others soon afterward isolated organisms from tobacco and in general supported Suchsland's hypothesis. These workers showed that a variety of organisms were present, in what were considered large numbers, i.e., as high as 112,500 bacteria and 12,500 fungus spores on 100 cm^2 of freshly fermented leaf.

Suchsland's bacterial theory soon fell into disrepute under a vigorous though not convincing attack by Loew (*12*) in 1899. Loew not only claimed that bacteria were not present in sufficient numbers to influence fermentation, but that sufficient moisture was not normally present for their development, and that even if sufficient moisture were present, bacteria do not find tobacco a congenial medium for growth.

An even stronger argument against the bacterial hypothesis, however, was the enzymic theory of tobacco fermentation developed by Loew and treated in further detail in two succeeding papers (*13*, *14*). This theory ascribes tobacco fermentation to oxidizing enzymes normally present in all living material, as oxidase, peroxidase, and catalase, the latter being present in dried, cured, and fermented tobacco, even after several years. The chief role in fermentation was first ascribed to peroxidase, an enzyme readily identified by its reaction with tincture of guaiacum in the presence of hydrogen peroxide. Loew's theory has since been supported mainly by Boeckhout and Ott de Vries (*2*) and Jensen (*5*), but very little new evidence to support or controvert the enzyme theory has appeared in the literature. Jensen (*6*) in 1915 used the Dewar-flask method of study and concluded that fermentation of leaf tobacco cannot be inhibited through the addition of chemicals detrimental to micro-organisms. The data presented are not clear on this point, however, and the variability of the temperatures in the incubator used was such as to render the data of doubtful value. On the other hand, Jensen suggests the existence of two types of fermentation, namely, one which proceeds at a moisture content of 20 percent or below and another which requires a higher moisture content. Recent investigations in Russia, discussed in considerable

It is evident from the literature that the determination of the nature of tobacco fermentation is complicated by the problem of what constitutes true fermentation and by the variation in the practical requirements of different types of tobacco.

Studies of a related nature have been conducted by many investigators on the spontaneous generation of heat in hay, straw, silage, manures, etc. The purely chemical, enzymic, and microbial explanations have all had stanch supporters, but it is interesting to note that recent investigations support the microbial theory (*16, 18*), at least under temperature and moisture conditions under which organisms will multiply, and even discount the cooperation of enzymes (*16*).

FIGURE 1.—A simple method for preparing tobacco samples for fermentation studies with pure cultures. The sterilized tobacco in the cellophane roll (*A*) may be inoculated at any desired points by inserting the syringe (*B*) through the cellophane before the roll is placed in the wide-mouth Dewar flask (*C*).

MATERIALS AND METHODS

At first the present studies were conducted with the ordinary narrow-mouth 1-quart thermos bottles. Later, wide-mouth flasks were secured which were easier to fill and permitted of handling the tobacco under fairly satisfactory aseptic and pure-culture conditions when desired. This was accomplished by first placing the tobacco in moisture-proof cellophane containers, sterilizing it with heat, and inoculating it with water suspensions of cultures of organisms by means of a Luer syringe inserted through the cellophane at one or more points. The cellophane containers were at first made the desired shape and size before being filled with tobacco; inoculations were then made by injections at a large number of points (fig. 1).

However, more even distribution of inoculum could be secured by

loosely and into which the inoculum was injected with a syringe and mixed with the tobacco by turning and agitating it. The tobacco was then pressed into one end of the bag, which was rolled into form to fit the wide-mouth Dewar flask (fig. 2).

It was found that the tobacco could be adequately sterilized in these containers without apparent physical injury to the leaf or any appreciable change in moisture content by placing the roll or bag in a sealed copper container which was placed in an ordinary steamer. Forty-five minutes of steaming, during which the tobacco reached a temperature of 80° C., was sufficient to prevent subsequent thermogenesis, and plating out showed that no organisms were present. In practice,

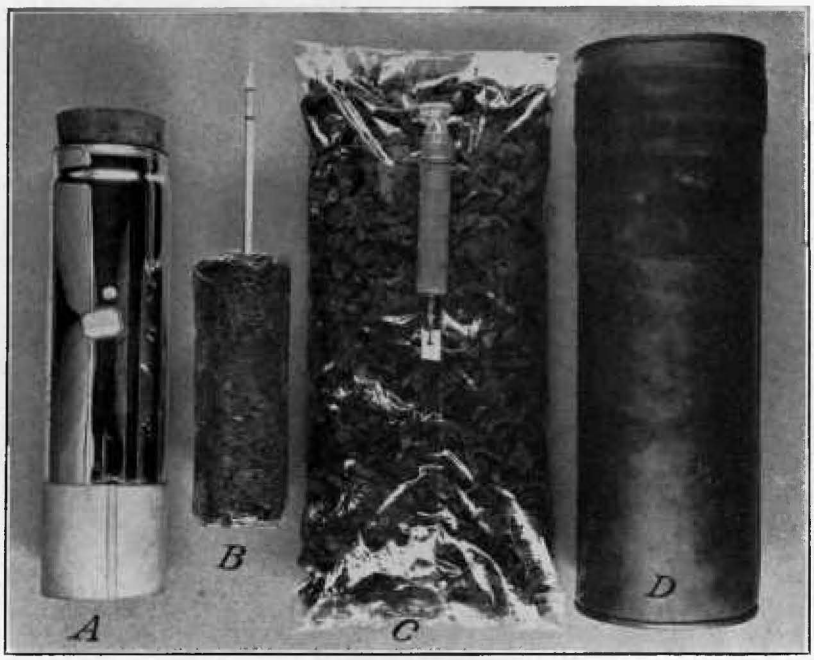

FIGURE 2.—The cellophane-bag (*A*, *B*, *C*) method permits of a uniform application of inoculum under aseptic conditions; the tobacco in the cellophane bag is sterilized in a copper, rubber-tube-sealed container (*D*), without undergoing any change in percentage of moisture.

however, the steaming was allowed to continue for 1 hour, during which time the tobacco reached a temperature of at least 85°. At these temperatures peroxidase was destroyed. In other tests peroxidase was found to be destroyed in water extract if heated for 10 minutes at 83°, and Loew (*12*) reports that the enzyme is destroyed at 87° in 3 minutes.

A limited amount of aeration was made available to the tobacco by various means. In the case of the narrow-mouth bottles a pliable perforated lead tube extended to the bottom of the flask. In the case of the cellophane containers, aeration was only provided by puncturing the cellophane at several points with the syringe or a hot needle and by not sealing the mouth of the flasks tightly. No other provisions for

say that the amount of oxygen available was equal to that in normal fermentation in large boxes or bulks. At least, preliminary trials with air aspirated through the bottles did not appreciably increase thermogenesis, whereas the replacement of the air with nitrogen, followed by sealing, greatly reduced the thermogenic power.

The tobacco used in the tests was largely of the local variety known as Havana No. 142, grown on the Wisconsin Experiment Station farm. This tobacco contained approximately 30 percent moisture, which is close to normal for Wisconsin tobacco in the bale soon after stripping. The leaves were first stemmed and cut into strips about one-fourth inch in width; this made the pack more uniform and easier to handle. One hundred and fifty grams (about 5½ ounces) of this

FIGURE 3.—One of the constant-temperature incubators and the thermos bottles used in the earlier experiments.

tobacco was usually used in the 1-quart thermos bottles. The moisture content was usually raised to 35 percent or more by atomizing the tobacco with the desired quantity of distilled water. In most instances the chemicals were applied with the water.

As soon as the bottles were filled they were placed in automatically regulated constant-temperature chambers. Most of the experiments were run in a large 30° C. incubator in which the temperature normally varied less than 1° (fig. 3). More significant results would no doubt have been secured in some cases by incubating at a somewhat lower temperature. Readings were taken at 24-hour intervals (i.e., at 9 a.m.), usually over a period of 10 days. The temperature increases shown in the tables were then based on the average of the incubator readings subtracted from the average of the flask readings over the 10-day period. The maximum increases, which usually occurred about the fifth day, were considerably higher than the

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