# STUDIES ON THE FERMENTATION OF TOBACCO. PART II—A STUDY OF VARIATIONS IN FERMENTATION PROCEDURE AND ITS EFFECT ON TOTAL PARTICULATE MATTER AND BENZO (A) PYRENE.

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Cigar leaf tobacco (**Nicotiana tabacum** L.) was fermented in the laboratory with an application of **Saccharomyces cerevisiae** or **Candida pseudopicalis** or without either at 30°C or 40°C. The following results were obtained.

The tobacco samples were well fermented at 30°C by the multiplication of bacteria.

The addition of **Saccharomyces** or **Candida** resulted in a rise in the number of bacteria during the early stage of fermentation.

Total particulate matter and benzo(a)pyrene content in the smoke of the fermented tobacco were not decreased positively by the fermentation treatment.

## INTRODUCTION

Most cigar tobaccos (*Nicotiana tabacum* L.) undergo fermentation prior to their use in smoking products in order to develop typical smoke taste and aroma (1). Many theories have been advanced to explain the nature of the fermentation process (2,3,4). In the view of these authors, Reid and coworkers (2), Jensen and Parmele (3) have shown that the major changes in fermentation are brought about through the activity of certain bacteria. Many attempts have been made to improve tobacco quality by the addition of certain microorganisms. Gribbins (5) studied the effects of bakers' yeast on tobacco fermentation and Giovannozzi (6) analyzed the changes in the nitrogenous compounds subjected to fermentation which was induced by some bacteria.

Recently, much attention has been paid to the problem of smoking and health. In the present experiment, cigar tobacco was subjected to fermentation induced by addition of *Saccharomyces* or *Candida*, at 30°C or 40°C, and total particulate matter and benzo(a)pyrene in the smoke were determined.

## EXPERIMENTAL METHODS

### Fermentation Procedure

A Japanese domestic variety "Nambu" leaf, which is a cigar wrapper type, was used in the experiment. *Saccharomyces cerevisiae* IAM 4552 or *Candida pseudopicalis* IAM 4843 was subcultured in a medium (Difco; malt extract, 3.0%) at 30°C for three days and a 100-ml aliquot was centrifuged to collect the culture cells. The cells were dispersed in 400 ml of sterile water and the suspension spread uniformly over the leaf in a basket (30 cm in diameter). Each basket had two kg of the leaf. The control basket received 400 ml of sterile water without organisms. The original moisture content of the leaf was approximately 35%. The baskets were placed for six weeks in either of two fermentation chambers, one of which was kept at 30°C and the other at 40°C. The relative humidity in the chambers was maintained at 80-90%.

Samples were taken every week for the determination of pH, moisture content and bacterial counts. The number of bacteria and pH were determined as described previously (8).

After six weeks, the leaf was removed from the chambers and manufactured into cigarettes for the determination of total particulate matter and benzo(a) pyrene.

### Total Particulate Matter (TPM)

Nine cigarettes, each 70 mm long, were smoked in an automatic smoking machine to a 30-mm butt length. A standard 35 ml puff of two-second duration was taken at one minute intervals. The smoke was collected on Cambridge filter pads and the increase in weight taken as TPM.

### Benzo(a)pyrene (BaP)

Benzo(a)pyrene was determined by the method of Davis (7) with a little modification. Glass-distilled solvents were used throughout the analytical procedure. Twenty cigarettes were smoked using the conditions described for TPM determination. The smoke condensate was collected in a glass trap which was cooled in a dry ice-methanol bath and dissolved in 100 ml of methanol-water (6:1). The suspension was extracted with hexane (3x50 ml). The hexane extract was evaporated to dryness in vacuo at 30°C and the residue was dissolved in 50 ml of hexane. The hexane solution was extracted with acetonitrile (5x40 ml). The combined acetonitrile extract were quickly evaporated to dry-

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Table 1. Moisture content (%) of the tobacco samples during fermentation

| Treatment | weeks 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| 30°C Control | 31.5 | 31.2 | 30.6 | 31.2 | 27.4 | 28.2 | 29.9 |
| 30°C Saccharomyces | 34.1 | 36.7 | 33.6 | 34.2 | 33.2 | 31.3 | 29.6 |
| 30°C Candida | 38.3 | 34.3 | 36.5 | 33.8 | 32.8 | 30.8 | 29.6 |
| 40°C Control | 35.3 | 35.2 | 31.7 | 32.3 | 33.2 | 31.4 | 27.2 |
| 40°C Saccharomyces | 34.3 | 31.6 | 30.0 | 31.6 | 28.3 | 26.5 | 28.0 |
| 40°C Candida | 38.0 | 34.1 | 37.4 | 32.1 | 26.6 | 29.8 | 30.1 |

Table 2. pH of the tobacco samples during fermentation

| Treatment | weeks 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| 30°C Control | 6.5 | 7.0 | 7.0 | 7.2 | 7.1 | 7.1 | 7.2 |
| 30°C Saccharomyces | 6.5 | 7.5 | 7.5 | 7.4 | 7.4 | 7.5 | 7.4 |
| 30°C Candida | 6.5 | 7.5 | 7.5 | 7.3 | 7.3 | 7.4 | 7.2 |
| 40°C Control | 6.5 | 6.4 | 6.9 | 6.7 | 6.8 | 6.9 | 6.9 |
| 40°C Saccharomyces | 6.5 | 6.6 | 7.0 | 6.9 | 6.7 | 6.9 | 6.8 |
| 40°C Candida | 6.5 | 6.5 | 7.0 | 6.9 | 6.8 | 7.0 | 6.9 |

Table 3. Total particulate matter and benzo(a)pyrene in the smoke of fermented tobacco

| Treatment | cigarette weight (mg) | no. of puffs | TPM (mg/ cigarette) | BaP (ug/100 cigarettes) |
|---|---|---|---|---|
| Untreated | 641 | 6.3 | 24.8 | 1.5 |
| 30°C Control | 833 | 7.5 | 26.4 | 1.7 |
| 30°C Saccharomyces | 799 | 7.5 | 25.4 | 1.7 |
| 30°C Candida | 805 | 7.8 | 27.0 | 1.8 |
| 40°C Control | 800 | 7.5 | 26.1 | 1.6 |
| 40°C Saccharomyces | 800 | 7.5 | 27.3 | 1.7 |
| 40°C Candida | 823 | 7.8 | 29.2 | 1.7 |

ness at 40°C. The residue was dissolved in five ml of hexane. The hexane solution was placed on a silicic acid column (13 mm x 20 cm; Mallinckrodt, 100 mesh) which was eluted with hexane and 20 ml fractions were collected. Each fraction was evaporated almost to dryness and streaked on acetylated paper (Car Schelicher & Shull, No. 2043). After the paper was developed with ethanol-water-toluene (17:4:4), the band containing benzo(a)pyrene was examined by UV light and extracted with methanol. The methanol solution was evaporated to dryness and 5.0 ml of methanol was added to the flask. The fluorescence spectrum of the solution was obtained using a Hitachi Spectrofluorometer set at an excitation wavelength of 376mμ The emission spectrum of an isolated compound in methanol was nearly identical with that of authentic BaP (**Figure 1**). Recovery of added BaP was 70-80% with this method and the analyses of duplicate samples were in good agreement. The fluorescence maximum at 405 mμ was read and the concentration of BaP was determined from the standard curve.

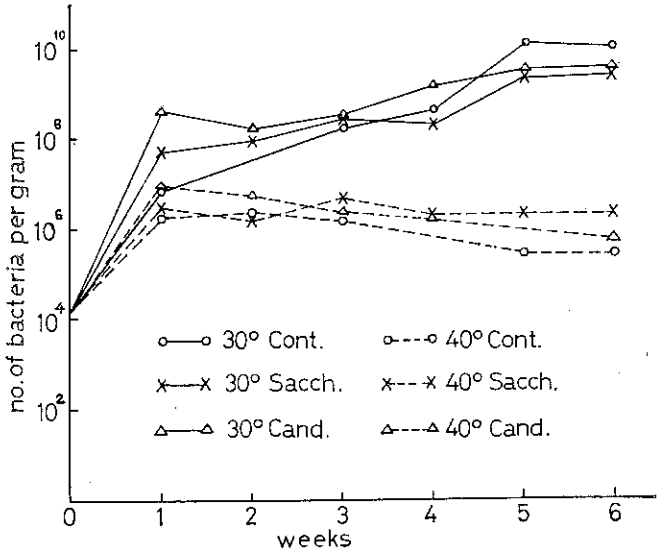


Figure 1. Fluorescence emission spectra of the isolated compound (dotted line) and authentic benzo(a)pyrene (solid line).

## RESULTS AND DISCUSSION

### Fermentation

The changes in moisture content and pH of the leaf during fermentation are shown in **Tables 1** and 2, respectively. The number of bacteria is shown in **Figure 2.**

The moisture content prior to fermentation was approximately 35%. After fermentation for six weeks, it had dropped to 27-30%. In the fermentation, the pH value of the leaf, which was originally 6.5, increased to 7.0-7.5 at 30°C, whereas at 40°C, the pH-increase was less. The final bacterial count of the leaf in the fermentation at 30°C reached $10^9$-$10^{10}$ per gram of tobacco, while at 40°C the count was only around $10^6$. In no case, was yeast detected on the leaves one week after the treatment or later. Few fungilor molds were detected.

These results suggested that the increase in the pH value of the leaf was related to the multiplication of bacteria. In the commercial bulk fermentation of "Nambu" leaf the bacterial count was 3 x $10^7$ at maximum and the pH value had a tendency to decrease slightly (8). In the present experiment, the leaf seems to have been well fermented by the multiplication of bacteria at 30° C. However, in the 40° C-fermentation, the maximum number of bacteria observed was too low to interpret that bacterial fermentation took place. The addition of *Saccharomyces* or *Candida* resulted in an increase in the number of bacteria during the early stage of fermentation as shown in **Figure 2.** The results are in accordance with those of Gribins et al (5).

The flavor and aroma did not seem to be improved particularly by the fermentation treatment or by the addition of *Saccharomyces* or *Candida*, when judged from a sensory test. However, the flavor, aroma and smoking quality were a little improved by the 40° C-fermentation.

### TPM and BaP of Smoke

Cigarettes having 55 ± 5 mm $H_2O$ pressure drop were used for the determination of TPM and BaP. The TPM values of the smoke of the fermented tobacco are shown in **Table 3.** The tobacco leaf before fermentation was also manufactured into cigarettes to compare TPM and BaP values with those of treated samples. The amounts per cigarette for the treated samples were somewhat higher than those for untreated ones. On the other hand, the weights of the untreated tobacco cigarette were significantly lower than those made from the treated tobacco. Moreover, the dry weight of the tobacco was decreased by nearly 20% of the original value during the fermentation. Thus, if the TPM values are calculated per unit weight of tobacco, those from the fermented samples are all lower than those of the untreated tobacco. The addition of *Saccharomyces* or *Candida* does not seem to have influenced the TPM values compared with those of the controls.

The BaP contents also are shown in **Table 3.** The values per cigarette for the treated samples were slightly higher than those for the untreated ones. However, as was mentioned above in the TPM discussion, the BaP values of the fermented samples tend to be significantly lower than untreated ones if the values are calculated per unit weight of tobacco.

## SUMMARY

Cigar leaf tobacco (*Nicotiana tabacum* L.) was fermented in the laboratory with an application of *Saccharomyes cerevisiae* or *Candida pseudopicalis* or

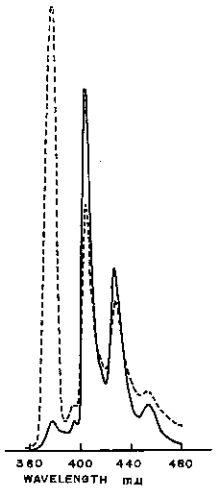


**Figure 2. The number of bacteria on tobacco samples during fermentation.**

without either at 30°C or 40°C. The following results were obtained.

The tobacco samples were well fermented at 30°C by the multiplication of bacteria.

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Total particulate matter and benzo(a)pyrene content in the smoke of the fermented tobacco were not decreased positively by the fermentation treatment.

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