

Role of yeasts in aroma development and modification of tobacco

In 1970 Manufacturing Department of Imperial Tobacco Products Ltd., expressed an interest in elucidating the nature of the fermentation of Club chewing tobacco with a view to accelerating as well as modernizing the fermentation procedure. Consequently, attempts were made to ferment Club chewing tobacco under laboratory conditions in a water-jacketed stainless steel container and these proved successful. The product obtained compared favourably with the regular product in colour, texture and aroma (1).

A study of the microbiology of fermented Club chewing tobacco (1) revealed that bacteria did not play any role. Bacterial counts of $20-70 \times 10^3$ obtained on nutrient agar remained constant throughout the fermentation. No moulds were detected when dilutions of chewing tobacco containing extract from up to 0.1 gm chewing tobacco were examined. On the other hand, yeast counts made on Wort agar (pH 4.8) showed that the number of yeasts increased as the fermentation progressed and then decreased again towards the end of the fermentation. Initial counts of $3-5 \times 10^3$ per gm. were found to increase to $20-50 \times 10^6$ per gm. Also, only yeasts grew on other media such as vegetable juice (V-8), tomato juice, and Bacto Micro Assay Culture Agar used to isolate lactobacilli, as well as under anaerobic conditions.

In order to confirm the role of the yeasts in aroma development, several of them were obtained in pure culture by standard procedures. An acceleration of fermentation was attempted by adding large numbers of yeast cells at the start of the fermentation. Two yeast isolates, numbers 5 and 6, were grown in Wort broth, and the cells harvested were inoculated into 15 lbs batches of cased unfermented chewing tobacco. Both isolates had no effect on the fermentation rate. However, when these isolates were harvested from a medium prepared from chewing tobacco itself, one of them (isolate no. 6) appeared to be very active in accelerating the fermentation. Overnight the chewing tobacco acquired the colour and aroma that was obtained after about 5-6 days of regular fermentation.

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On account of the remarkable effects this yeast isolate had on the aroma of chewing tobacco, it occurred to us that, perhaps, it would have a similar effect on other tobacco products. Consequently, we decided to find out what effect addition of this yeast would have on the aroma of flue-cured tobacco. The yeast (isolate no.6) was grown in flasks in a 10% flue-cured tobacco extract, 0.1% yeast extract medium with agitation on a rotary shaker. The cells were harvested by centrifugation and a 0.012% suspension prepared. 1900 ml. of this suspension were added to about 2.25 kgm. of flue-cured tobacco, and the mass was tightly packed in a large container. A control was kept in which 1900 ml. of water was added to about 2.25 kg. tobacco. Tobacco samples were drawn immediately after packing (0 hr.) and 1, 2, 3, 4, 5 and 7 days later, and yeast counts made on Wort agar. Portions of these samples were dried at ambient temperature and used for determination of reducing sugars. Dried samples were also ground and made into cigarettes by a procedure developed in this laboratory (2). Samples drawn on the 5th day were dried and cut at 45 cuts per inch for making cigarettes.

As in the case of chewing tobacco, addition of yeast suspension to flue-cured tobacco at 50% moisture content resulted in the tobacco acquiring a pleasant aroma. Interestingly, the control flue-cured tobacco (no addition of yeast cells) held at 50% moisture level, too, at the end of three days developed an aroma similar to that in the yeast treated tobacco. A microbiological examination of the control tobacco revealed the presence of large numbers of yeasts after the third day (see table 1). There was no possibility of cross contamination, and it was due to the storage conditions of the control tobacco which led to the rapid multiplication of the yeasts initially present. However, it must be mentioned here that such a selection of yeasts under the storage conditions of the control tobacco is erratic and is often accompanied by significant mould growth due to leakage of air into the container or the polyethylene bag. In this respect, treatment of tobacco with yeast cells harvested from a tobacco extract medium not

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only results in a much more rapid change, but also eliminates any mould problems, which, of course, could also be eliminated by making the storage completely anaerobic. In a similar experiment in our laboratory in which rapidly dried green tobacco was held at 50% moisture content in polyethylene bags it also resulted in yeasts dominating the ensuing fermentation.

Thus, it appears, that when conditions of high osmotic pressure and low oxygen tension (or anaerobic) prevail in tobacco, yeasts are selected out to the exclusion of all other organisms, a phenomenon perhaps first observed in the 1830's during a shipment of Connecticut leaf across the Atlantic to Germany, and still something of a mystery to many tobacco farmers and agriculturists (3). On the other hand, some recent studies in this laboratory on the silaging of freshly harvested green tobacco as well as such a tobacco that has been wilted/yellowed, and which have moisture contents of 85% and 65% respectively, suggest that under conditions of low oxygen tension (or anaerobic) growth of microorganisms, presumably belonging to the family Lactobacillae, occurs.

A growth of yeasts, similar to that occurring in the control tobacco referred to earlier, appears to have occurred in studies carried out in the research laboratories of Brown & Williamson (4, 5, 6), as well as in a Korean study (7). In the Louisville study, undried tobaccos were stored at moisture levels ranging from 22-28% and anaerobic conditions, with and without yeast (Saccharomyces cerevisiae grown in ordinary sugar media) treatment. In this study too, the water treated samples acquired an aroma, comparable to that acquired by flue-cured tobacco aged for long periods, but different from the aroma characteristics of their yeast treated samples. We would suspect that this aroma development in the water control tobacco was due to the growth of yeasts. We do not know whether or not the Louisville workers carried out a microbiological examination of their water treated control tobacco.

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It is not surprising that the Louisville workers found that their yeast treated flue-cured tobacco was different in aroma characteristics from the water treated control. They harvested their yeast cells (baker's yeast) from regular sugar media on account of which no synthesis of inducible enzymes, capable of utilising other tobacco components, occurred, which was perhaps the case in their controls, in which yeasts, if they grew, did so on tobacco components. In our experiments we used a culture (as yet unidentified), initially isolated from a tobacco product in which it was found growing, and which for our experiment was harvested from tobacco extract medium. Thus, in our case, both the yeast treated as well as control tobaccos appeared to have acquired a similar aroma. It appears to us that the yeast is similar to the one growing in the control tobacco, as well as similar to the one we have found growing in the fermentation of a rapidly dried, freshly harvested green tobacco referred to earlier.

In the Korean study a nicotine-resistant Hansenula yeast was used to ferment flue-cured tobacco. Forced aging with and without yeast inoculation gave a tobacco that was comparable to a flue-cured tobacco that had been aged more than 2 years. Bacterial and yeast counts increased upto about 15 days of aging and then decreased, and this increase in yeast population could account for the tobacco acquiring the properties of aged tobacco. However, it is difficult to reconcile the reported increase in total sugars with the increase in microbial population, and only when details of this study are available will it be resolved.

Both our yeast and water treated tobaccos were dried, cut at 45 cuts per inch and made into cigarettes. No systematic subjective evaluation of the smoking properties of these was undertaken, consequently the results obtained are inconclusive. However, preliminary smoking studies by members of the research laboratory tend to favour both the yeast and water treated tobacco cigarettes over regular flue-cured

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cigarettes. To some these cigarettes appeared to be milder and less irritating to the throat, while others found no difference between them and regular flue-cured cigarettes. The above reactions to these cigarettes is surprising, mainly on account of the fact that at least the yeast treated cigarettes contain less than 6% sugar (see table), and it is the sugar in flue-cured that is believed responsible for their smooth taste and flavour. From the table it will also be seen that yeast treatment also modifies the tobacco sufficiently as to give smoke of lower DCIP-reducing activity (2). This is significant from the point of view of one of the main objectives of the research department which is the modification of yield and chemical composition of tobacco smoke. We have employed the DCIP-reducing activity of smoke extensively to monitor such modifications.

In conclusion, it is this author's opinion that studies such as these, particularly with respect to the role of induced enzyme synthesis in microorganisms used to modify tobacco, are worthwhile undertaking if tobaccos with modified aromas and/or lower biological activities are to be developed. Such studies may also prove valuable in improving flavour and taste of tobacco substitutes.

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August 15, 1974

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TABLE I
DCIP-REDUCING ACTIVITY OF AND YEASTS IN
"FERMENTING" FLUE-CURED TOBACCO

Addition to flue-cured tobacco	Incubation period in hours	Reducing sugar content %	Yeast counts on Wort agar x10 ⁶	DCIP-reducing activity
				Specific activity - "Units" per mg. TPM
Water	0	23.8	0.016 *	7.34
Yeast	0	24.3	320.0	7.33
Water	24	25.3	0.037 *	7.10
Yeast	24	17.1	460.0	6.45
Water	48	25.3	0.049	7.04
Yeast	48	13.6	350.0	6.33
Water	72	25.0	5.0	6.69
Yeast	72	11.3	410.0	5.94
Water	96	24.9	80.0	6.82
Yeast	96	7.5	280.0	5.40
Water	120	21.5	170.0	6.60
Yeast	120	5.7	120.0	5.31
Water	168	13.1	330.0	5.77
Yeast	168	5.7	44.0	5.08

* Mostly sporeforming bacteria

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