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MICROBIOLOGICAL OXIDATION OF ETHANOL IN VOLATILE FRUIT CONCENTRATES [1]

Production of volatile fruit concentrates, or essences, developed at this laboratory, Milleville and Eskew (1944), leads frequently to an accumulation of ethyl alcohol exceeding 0.5 per cent in the distillates, thus making them subject to regulations by the Alcohol Tax Unit.[3]

The problem, therefore, was to remove ethanol—not an essential flavoring agent—from a mixture of many closely related compounds responsible for the characteristic fruit flavor. Of several possible ways to accomplish it, the following were tried: Alcohol precipitants, such as 3,5-dinitrobenzoyl chloride, Lipscomb and Baker (1942), did not give a reaction with ethanol in the extreme dilutions of the experiment. A crude preparation of alcohol dehydrogenase from the sprouts of oats *(Avena sativa),* Berger and Avery (1943), gave unsatisfactory results because of low activity and instability of the enzyme. A purified alcohol dehydrogenase system could not be considered for commercial use because of the cost. The most promising results were obtained by using the alcohol dehydrogenase contained in *Acetobacter.* In this report, the general principles are established for the treatment of fruit concentrates with resting cells of the bacteria. Many details have to be worked out to make the process commercially feasible, but since the work cannot be continued at present it was thought advisable to present the following preliminary results.

MATERIALS AND METHODS

Apple Essence: The commercial preparation of fruit essence is a two-step process. The juice is flash-vaporized to remove about 10 per cent of the volume, carrying with it substantially all the volatile flavor ingredients. These vapors are then passed to the bottom of a fractionating column, where a further 15-fold concentration results, or 150-fold for both stages. Ordinarily, the vapors from the first step are not condensed. In the present procedure they are condensed, with the idea of so controlling the fold at this stage that the condensate will have less than 0.5 per cent ethanol. This condensate is subjected to the action of *Acetobacter,* and then the product is revaporized and fractionated to complete the second step.

The essence is a colorless aqueous solution of the volatile constituents of natural apple flavor. The flavoring substances comprise from 0.5 to 0.9 per cent of a 150-fold essence. About 24 constituents have been identified,

[1] Report of a study made under the Research and Marketing Act of 1946.

[2] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

[3] Federal Register 14, 5869-5879 (1949).

including compounds closely related to ethyl alcohol, such as methanol, propanol, butanol, acetaldehyde, and acetone, White (1950).

Organisms: Several species of *Acetobacter*[4] were found capable of metabolizing ethyl alcohol in extremely low concentrations. *Acetobacter acetosum,* NRRL B-468, and *Acetobacter rancens,* NRRL B-65, produced abundant growth on media containing alcohol as the only source of energy, and the washed bacteria had considerable enzymic activity. But difficulties in collecting and washing the bacteria, with their slow sedimentation rate, necessitated the search for another organism. *Acetobacter aceticum,* NRRL B-43, was selected because of its ability to produce, under certain conditions, sponge-like growth which greatly facilitated its harvesting and separation from the nutrient constituents. In addition, it possessed a sufficiently active alcohol dehydrogenase. Surface membranes of *A. aceticum* are thick and leathery and contain cellulose. The organism closely resembles *Acetobacter xylinum* or is even identical with it.[5]

Culturing A. aceticum: The bacteria were grown with shaking in one-liter Erlenmeyer flasks containing 250 ml. of a medium consisting of 0.5 per cent tryptone, one per cent meat extract, 0.2 per cent sodium chloride, and two per cent glucose. After two days the shake culture served as inoculum for 250-ml. Erlenmeyer flasks with 25 ml. of medium containing three per cent alcohol instead of the glucose. The surface pellicles were allowed to develop undisturbed to a thickness of about five mm., taking four days at 28°C. (82°F.). The leathery membranes were used to inoculate the 10-liter batches of submerged, aerated cultures. A 22-liter round-bottom flask was fitted with a powder funnel, an air outlet, and an air inlet, which ended at the bottom of the flask in a perforated bulb of one-inch diameter. Each flask contained 10 liters of the following medium: one per cent meat extract, 0.5 per cent peptone, 0.2 per cent NaCl, three per cent $CaCO_3$ as marble chips, and, after sterilization, three per cent alcohol. Each flask was inoculated with 20 pellicles of 70-mm. diameter, which were added through the powder funnel. To promote formation of large pieces of connective membranes, the aeration was started slowly and increased after from three to four days, when sponge-like growth was well developed. The cultures were harvested after 10 days, filtered through gauze, and washed three times. For each washing, the membranes were immersed in ice-cold water for one-half hour and filtered off again. A uniform suspension of bacteria was obtained by cutting the connective growth in a Waring Blendor. Aliquot parts of the suspension were taken for dry weights and dehydrogenase determinations. The average yields of *A. aceticum* growth in deep cultures were three gm. of bacteria (dry weight basis) for 10 liters of medium. The rather cumbersome procedure of still cultures in Fernbach flasks, with 250 ml. of medium, produced yields four times larger.

The dehydrogenating ability of the bacteria was determined by the Thunberg technique, with ethanol as substrate and methylene blue as hydrogen acceptor at 30°C.(86°F.) *in vacuo.* Each Thunberg tube contained

[4] Drs. R. W. Jackson and R. T. Milner of the Northern Regional Research Laboratory kindly supplied the cultures of *Acetobacter.*

[5] Dr. R. T. Milner, personal communications.

0.1 M ethyl alcohol, 0.005 M acetate buffer pH 5.6, 0.0001 M methylene blue, and the washed bacteria, in a total volume of 2.5 ml. Average time required to reduce 80 per cent of the methylene blue with different amounts of bacteria is shown (Fig. 1). Direct conclusions from the reduction time of methylene blue to the speed of alcohol oxidation in air or oxygen were not possible, since the rate of dehydrogenation with methylene blue as hydrogen acceptor is considerably lower than with oxygen, and leuko methylene blue exhibits an inhibitory effect, Wieland and Bertha (1928). But the method provided sufficient information about the relative strength of alcohol dehydrogenase in resting cells of *Acetobacter* to serve as a guide for the application of an appropriate amount of bacteria in the oxidation process.

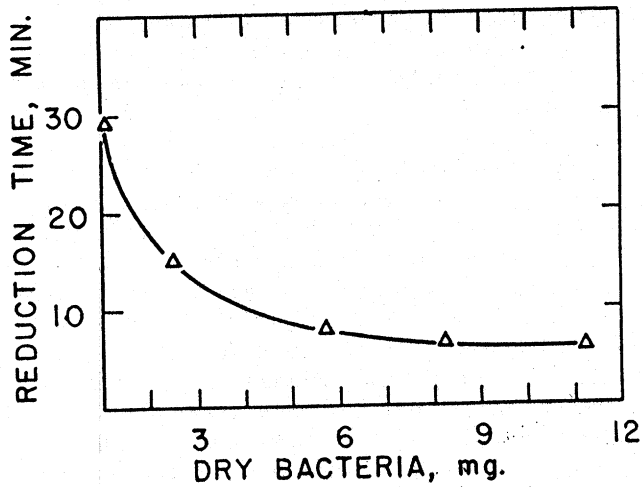

FIG. 1. Alcohol dehydrogenase activity of *A. aceticum* as determined by the time required to reduce 80 per cent of methylene blue at 30°C. (86°F.) in the presence of ethyl alcohol and different amounts of bacteria.

Washed bacteria were used for the alcohol oxidation to prevent introduction of foreign and off-flavor substances in the essence. Though the chosen species of *Acetobacter* should be capable of oxidizing ethanol to completion, that is, to carbon dioxide and water, the resting bacteria carried dehydrogenation only to the acetic acid stage under the conditions of the experiment. Addition of nicotinic acid, Kligler and Grossowicz (1941), or pyruvate, Tosic (1946), had no favorable influence on the acetate oxidation. Since aqueous or saline suspensions of the resting cells did not maintain full dehydrogenase activity for 24 hours, most of them were used immediately. If storage was unavoidable, they were centrifuged, frozen, and kept at —22°C. (—8°F.), with little loss of activity. No attempt was made to separate the enzyme system from the cells.

Alcohol Oxidation and Essence Treatment: To study the optimal conditions for the treatment of apple essence with bacteria, a mixture was prepared which simulated the composition of a possible condensate obtained in the first step in the production of essence, Milleville and Eskew (1944). The aqueous solution contained a 10-fold essence, three per cent apple juice,

and 0.4 per cent ethyl alcohol. It was necessary to conduct the oxidation in a close system, to minimize loss of the highly volatile flavor constituents. The volume of oxygen thus required was empirically determined to be five times the amount calculated for oxidation of ethanol to acetic acid. The operation finally adopted was as follows: Two-liter jars were each filled with 200 ml. of the oxidation mixture, which contained the washed bacteria and had been adjusted to the pH optimum (5.6) of the bacterial alcohol dehydrogenase. The jars were filled with oxygen, tightly sealed, and shaken at 30°C. (86°F.) with a speed of 90 strokes per minute. Before opening, they were cooled to 4°C. (39°F.) to prevent loss of essence.

Simpler procedures in open systems, such as blowing air through the oxidation mixture in the presence of the bacteria, or a continuous process similar to the quick vinegar process, gave excellent alcohol oxidation but had to be discontinued because of almost complete loss of the volatile constituents.

After it had been established that the essence in no way interfered with alcohol oxidation, a solution containing 0.4 per cent alcohol and three per cent apple juice was used for most experiments undertaken to determine the limiting amount of bacteria and time required for complete oxidation. To detect how the organoleptic properties of the essence are affected by the bacterial treatment, rather extreme conditions were chosen, with excessive oxidation periods of from 15 to 20 hours. To operate the essence recovery apparatus, a minimum of four gallons was required. Since for technical reasons only small volumes could be handled in the dehydrogenation process, 20- to 40-fold essences were submitted to the bacterial treatment and then diluted to a theoretical one-fold concentration with tap water, thus providing the required volume for the recovery unit. The oxidized material, with a pronounced odor of acetic acid, was neutralized to pH five and filtered, and the essence recovery procedure was carried out under the following conditions: Input rate of five gallons per hour, 10 per cent vaporization, and final concentration ratio of 100 to one (100-fold). The controls, consisting of one-fold solutions of untreated (alcohol-containing) essence, were submitted to the same concentration process.

Alcohol Determinations: At the beginning of this work, alcohol determinations were made by a specific gravity method adopted from the Association of Official Agricultural Chemists' Book of Methods, Sixth Edition, Paragraphs 15.4, 16.47, and 16.6. The method involved distillation, followed by measurement of specific gravity when no essence was present; salting, extraction with petroleum ether, and distillation of the aqueous alcoholic salt solution, followed by measurement of specific gravity when essence was present. When applied to solutions containing 0.40 per cent ethanol plus three per cent apple juice, this method gave an average value of 0.36 per cent, with a standard deviation of 0.11 per cent ethanol.

It was desirable to develop a method of greater precision for measuring low ethanol concentrations. Determinations of the refractive indices of the alcoholic solutions on a differential refractometer gave consistent results if certain precautions were observed in preparation of the samples. The differential refractometer, Brice and Speiser (1946), measures difference in refractive index directly with a limiting sensitivity of about 3×10^{-6}.

Because of its high sensitivity and its relative insensitivity to temperature change, it is much better suited to determination of ethanol in water, in the concentration range of 0.5 per cent or less, than the dipping or Abbé types of refractometer. The instrument was calibrated with standard solutions of from 0.5 to 0.05 per cent ethanol in water. Results of the measurements were precise to ± 0.01 per cent ethanol.

To prepare samples for the refractometer readings, 25 or 50 ml. of the oxidation mixture were adjusted to pH eight and distilled at the slow rate of 25 ml. per hour until almost the entire initial volume had been collected, the exact volume being restored by addition of water. A faster distillation resulted in slightly turbid solutions which could not be cleared and which were unsuited for the refractive index determinations. Since the essence and bacterial metabolites gave refractive indices of their own, they were compensated for by controls containing bacteria, essence, and apple juice, but no alcohol. The control samples were submitted to the oxidation procedure, then distilled, and the distillates measured in the differential refractometer. The difference beetween the values thus obtained for the samples and the corresponding controls was the apparent alcohol content of the sample. Comparable results were obtained either by using one half-cell of the apparatus for the sample and the other half-cell for the control, or by computing the readings of both sample and control against solvent.

RESULTS

Data obtained by the differential refractive index method made it possible to record the oxidation progress clearly and accurately (Table 1 and Fig. 2). Linearity of dehydrogenation versus time extended to about 75 per cent oxidation. Removal of the last 25 per cent of ethanol proceeded at an increasingly slower rate, requiring another two hours in the presence of an approximate one per cent bacterial suspension.

The active alcohol dehydrogenase content of *Acetobacter aceticum*, grown in deep or still cultures, varied little, following in general the curve given (Fig. 1). Resting bacteria of average activity, in one per cent concentration (on dry weight basis), completed the oxidation of 0.4 per

TABLE 1

Oxidation of 0.4 Per Cent Ethanol Solutions by A. aceticum in Closed Systems Under Oxygen at 30°C. (86°F.)[1]

Time of oxidation	Dry weight of bacteria	Essence present	Ethanol after oxidation
hr.	*pct.*		*pct.*
0.25	0.8	none	0.35
0.5	0.8	none	0.31
1	0.8	none	0.25
2	0.8	none	0.11
2	1.1	none	0.09
3	1.1	none	0.02
4	1.1	none	0.00
5	1.1	none	0.00
5	0.6	30-fold	0.00
16	0.6	30-fold	0.00

[1] Alcohol determinations by differential refractometer.

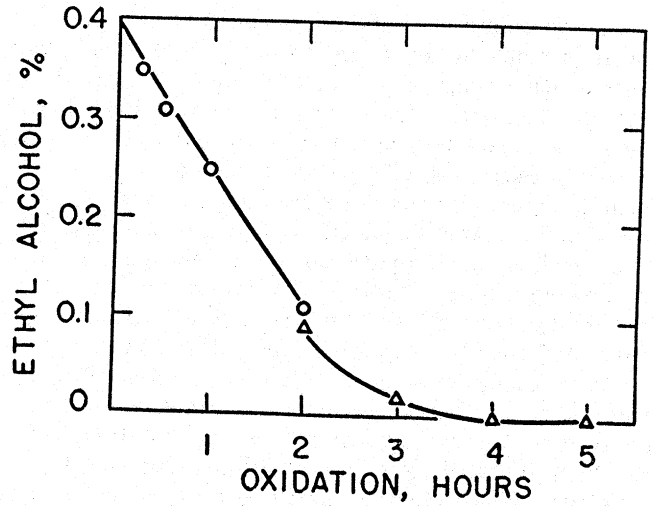

FIG. 2. Progress of oxidation of a 0.4 per cent ethanol solution by *A. aceticum* at 30°C. (86°F.)

O in presence of 0.8 per cent (dry weight) of bacteria.
Δ in presence of 1.1 per cent (dry weight) of bacteria.

cent ethanol solutions in four hours. Smaller amounts of bacteria may be used by extending the time of contact with the oxidation mixture. Possibly the same cells could be used twice. But exceedingly prolonged shaking of the essence at 30°C. (86°F.), even without bacteria, impaired the "freshness" in the aroma of the apple essence.

The essence, even in high concentrations, did not alter the enzymic activity. A 0.6 per cent bacterial suspension removed the alcohol completely in five hours from a 0.4 per cent ethanol solution, in the presence of 30-fold essence.

For optimal conditions of flavor retention, six to 10 gm. of bacteria should be used for one liter of essence of any concentration containing 0.4 per cent ethanol, over a contact period of from four to six hours. These proportions would require the growth of 20 liters of aerated cultures, or five liters of still cultures.

The recovered essences, of both treated samples and controls, were compared in respect to their concentrations and flavor changes. The concentration was judged by ascertaining the highest dilution at which the characteristic aroma was still detectable. Recovered essences from the bacteria-treated samples were of 45- and 41-fold concentrations, whereas strength of the control after essence recovery was 74-fold. This loss of about 40 per cent could probably be greatly reduced by using shorter oxidation periods and enlarging the facilities for the dehydrogenation process.

The over-all rating as to quality was obtained by adding the essences on the basis of their tested fold, to essence-free concentrated apple juice, Griffin, Talley, and Heller (1947). The reconstituted juices, containing either treated or control essences, were submitted to a taste panel of about 10 people. Flavor qualities were rated on a scale of one to 10, 10 repre-

senting excellent, and one an objectionable, flavor, Heller, Nold, and Willaman (1947, 1949). The alcohol-free essences received ratings of 6.1 and 5.9 against 7.3 and 6.8 for the controls. No odor of acetic acid was detectable in the treated products. Thus, both methods of evaluation indicate a loss of essence during the process as a whole.

CONCLUSIONS

It can be concluded that ethyl alcohol in concentrations below 0.4 per cent can be removed from apple essence by resting cells of *Acetobacter*. It does not seem, however, that the process would be commercially feasible in its present state of development. Rather large volumes of culture are required to furnish the bacterial cells for treating smaller volumes of essence. The time for treatment is several hours. There is some loss of essence. Now that the general principles are established, however, it is possible that more efficient details could be worked out, such as a more prolific or more active species of *Acetobacter*, a better medium, use of the cells a second or third time, more effective conditions for the oxidation of the alcohol, and reduction of the loss of essence.

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SUMMARY

Resting cells of *Acetobacter aceticum* are capable of removing ethyl alcohol from mixtures of chemically related substances. This property has been utilized for its elimination from volatile fruit concentrates. Apple essence of 20- to 40-fold concentration, containing 0.4 per cent ethanol, was freed of the alcohol by treatment with 0.6 to one per cent bacteria (dry weight basis) in a closed system under oxygen for from four to six hours.

The procedure caused some loss of volatile constituents but did not impair the character of the flavor. The same method could no doubt be used on any other volatile fruit concentrate.

A method is described in which a differential refractometer is used for ethyl alcohol determinations in concentrations of 0.5 per cent or less with a precision of 0.01 per cent.

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