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THE *AEROBACTER* FERMENTATION OF CUCUMBERS DURING SALTING

By J. L. ETCHELLS, F. W. FABIAN, and I. D. JONES

MICHIGAN STATE COLLEGE

AGRICULTURAL EXPERIMENT STATION

SECTION OF BACTERIOLOGY

East Lansing

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These studies represent reports on a cooperative project between the Bacteriological Section of the Michigan Agricultural Experiment Station; the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture; and the Horticultural Department of the North Carolina Agricultural Experiment Station.

PART I.

Bacteriological and Biochemical Studies of the Organisms Responsible for a New Type of Gaseous Fermentation in Cucumbers[1]

By J. L. ETCHELLS[2] and F. W. FABIAN[3]

INTRODUCTION

APPROXIMATELY 6,000,000 bushels[4] of pickling cucumbers are raised annually in the United States, representing a cash income of about $3,600,000 to the growers. Because only a small acreage, usually one to two acres, is planted per grower it is estimated that approximately 60,000 farmers are benefited by the pickling industry.

The commercial salting procedure generally used, briefly, is as follows: Large wooden vats of 400- to 1,000-bushel capacity are filled with green cucumbers and are fitted with a false head; then 40-percent saturated salt brine is added to a level a few inches above the head. The brine strength is increased according to a set schedule so that the salt concentration reaches 50 to 70 percent saturation by the end of the sixth week. An acid fermentation commences shortly after the cucumbers are brined and continues for from 2 to 6 weeks.

The fermentation which takes place is caused by salt-tolerant organisms. The initial inoculum of microorganisms for the fermentation comes from the cucumbers and from adhering particles of soil. They utilize as their nutritive material the soluble constituents that diffuse into the brine as the result of the action of salt on the cucumber tissue. The action of the microorganisms on the fermentable material in the brine brings about the production of various compounds (chiefly lactic acid, also acetic acid and alcohol) as well as the evolution of considerable quantities of gas.

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[1]A summary of the thesis presented by the senior author to the Graduate School of Michigan State College, June 1941, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Contribution 143, Agricultural Chemical Research Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

[2]Bacteriologist, Agricultural Chemical Research Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Raleigh, N. C.

[3]Research Professor of Bacteriology, Michigan Agricultural Experiment Station.

[4]Agricultural Statistics, U. S. D. A., Washington, D. C. (1939).

At the completion of the curing process (about 3 months), the cucumbers have changed from the green, opaque, air-filled, buoyant fruit to olive-colored, translucent, air-free salt stock.

It is evident that the curing process is one of complex bacteriological, chemical and physical changes. Any one of these changes constitutes adequate basis for considerable fundamental investigation in an attempt to gain a better understanding of the principles involved during curing. Such studies would help materially in placing the pickling industry in the group of scientifically controlled fermentations.

For the past 40 years research on cucumber fermentation has dealt principally with the bacteriology of the lactic acid fermentation, with a very small proportion of the work being carried out under plant conditions. Very little attention has been given to the fact that probably fermentations due to microorganisms other than the lactic acid bacteria occurred and could contribute to the general fermentation. That such is the case is evidenced by the fact that the true yeast fermentation, associated with the general cucumber fermentation, was not recognized by workers in this field until as late as 1940 (12). Similarly, combined bacteriological and chemical investigations upon cucumber fermentations at certain salt concentrations point toward the inclusion of still another phase in the fermentation proper. The latter phase is brought about by a group of gas-producing, salt-tolerant microorganisms and can be detected most easily by the evolution of gas which is composed of approximately equal parts of carbon dioxide and hydrogen.

The isolation, identification and physiological studies of this group of organisms as well as the observations dealing with their typical fermentation under plant conditions constitute the basis for this study.

HISTORICAL REVIEW

Until recently, bacteriological investigations on brine cucumber fermentations have dealt principally with that phase of the fermentation resulting chiefly in the production of lactic acid. As early as 1899 Aderhold (1) studied the acid fermentation in dill brines and found that it was fostered by anaerobic conditions and that such conditions resulted in the production of more acid. In 1909 Kossowicz (24) confirmed Aderhold's work and also studied the flora of the active fermentation in dill brines. In addition, experiments on the use of pure cultures for starters for dill pickle fermentation were performed. The observations of these workers, while conducted on dill brines, are particularly interesting and can be considered of fundamental significance.

LACTIC ACID FERMENTATION

Prior to 1913 there was little work published in this country on cucumber fermentation. In 1913, Rahn (29), in a bacteriological study of cucumber fermentations revealed that actively fermenting brine contained as many as 200,000,000 acid-forming bacteria per cc. and the acidity of the brine at the end of active fermentation, when calculated as lactic acid, reached 0.6 to 1.2 percent.

Brown (8), in 1916, in an abstract of an unpublished article, made a series of observations covering many aspects of cucumber fermentations. In reference to the production of acid, he describes the responsible microorganisms as short rods or cocci, arranged chiefly in chains and being facultative with respect to oxygen requirements. He further observed that the ratio of lactic to acetic acid was 2 to 1 and that other acids, propionic, butyric and benzoic, occurred in traces.

During the years 1919, 1920, 1922, Le Fevre (25, 26, 28) reported his observations on a series of experiments. He pointed out that cucumbers, as they came in from the field carried numerous bacteria, the principal groups being "lacto-bacilli," aerobic spore-formers, gas-formers, yeasts and molds. The "lacto-bacilli" were claimed to be the most tolerant of salt and were considered the most significant in acid production. The optimum temperature for the "lacto-bacilli" was found to be 30° C. The addition of fermentable sugar was reported to be advantageous in bringing about a quicker onset of the fermentation and resulted in a high degree of acidity. Le Fevre also stated that a higher acid content developed in the higher as compared with the lower salt content brines. Campbell (9) reported: (a) that the microorganisms responsible for the souring of milk were concerned with the cucumber fermentation; (b) that the lactic group of organisms should be supplied with oxygen; (c) that yeasts played a desirable role in the fermentation. Le Fevre (27) took issue with Campbell (9) because of the latter's misconception of the bacteriological changes during cucumber fermentations. A careful inspection of Campbell's report shows that he was undoubtedly confusing true yeasts with Mycoderma (false yeasts) from the heavy surface scum.

Tanner (35), 1926, in a discussion of the curing process, claimed that the brine acidity resulted mainly from volatile acids rather than lactic acid. He believed that while lactic acid was generally mentioned as a predominating acid, this was usually done without adequate chemical examination. The curing process was said to be chiefly an acid-gas fermentation. Fabian (13), in 1930, presented a general discussion of the consideration of pickle manufacture and referred principally to the observations of Le Fevre which have been discussed previously. Fabian also stated that the production of cucumber pickles was a scientific process and a thorough knowledge of the funda-

mentals was essential for successful manufacture. The influence of salt was stressed both because of the osmotic effect it exerted on the cucumbers and for its effect in encouraging lactic acid bacteria to the exclusion of putrefactive bacteria. During the same year Joslyn (23) presented a general article in relation to pickle manufacture. His discussion of the lactic fermentation was the same as that of earlier authors.

It is well to point out that the work on the acid fermentation of brined cucumbers up to about 1930 consisted mostly of general discussions and opinions as viewed by the various authors and directed toward the industry with very little bacteriological or chemical data included as supporting evidence for the conclusions made. However, the work definitely presents many interesting and valuable contributions to the general understanding of the bacteriological processes of brine cucumber fermentations. Tanner and Eagle (36), in an excellent general review of the literature through 1926, summed up the situation when they stated, "Despite the fact that this fermentation is an old one, much research may yet be done and must be done before it may be considered a controlled fermentation industry. There is a great need for combined microbiological and chemical investigations." They might also have added that there was a great need for investigations to be conducted under conditions which could be considered typical of the industry, a point which up until 1930 had not been given serious consideration.

Although a routine differential plating medium suitable for detecting acid-forming organisms (weak and strong) and also peptonizing bacteria was reported and described in great detail by Ayers and Mudge (4) in 1920, no adaptation was made of this medium to cucumber fermentations for 12 years. Fabian and co-workers (14), 1932, in perhaps the first systematic study of the microflora and chemical end-products of cucumber fermentations, used this medium to advantage in determining the populations of acid-forming organisms occurring in brines of initial strength of 8- and 10.6-percent salt concentration. In addition to the study of the effect of acids, bases and salts on the fermentation, chemical determinations were made during the fermentations with respect to total acids, volatile acids, alcohol and reducing sugars. The results of the investigations, in general, showed that the flora of the fermentation with respect to acid-forming organisms consisted chiefly of weak acid-formers. Also that there was a greater number of acid-formers in the weaker brines, and that they reached a maximum sooner than in the stronger brines. An examination of the chemical analysis showed that there were greater amounts of non-volatile acids, volatile acids, and alcohol in the weaker brines as compared with the stronger brines.

In 1935 Vahlteick, Haurand, and Perry (37) attempted one of the first bacteriological studies conducted under commercial conditions. They studied the microflora during the curing of cucumbers salted at a brine concentration of 10 percent. The data from their limited observations on two vats led them

to believe that the following groups of microorganisms were present: high-acid producers of the *Lactobacillus* type; low-acid producers of the *Leuconostoc* type; round yeasts; ellipsoidal yeasts (scum yeasts); spore-forming bacteria; and unclassified organisms. The formation of acid during fermentation was attributed to acid-producing organisms identified as *Lactobacillus cucumeris* and to two species of the *Leuconostoc* genus. Wüstenfeld and Kreip (41), 1933, divided the lactic acid bacteria into the following genera: *Bacillus, Streptococcus,* and *Pedococcus.* Furthermore, they stressed the desirability of the addition of pure cultures as starters subsequent to thorough washing of the cucumbers at the time of salting. Jones (21), in 1940, reported extensive studies over a 3-year period at a commercial pickling plant on the salting of cucumbers in barrels. Fermentations in initial brine concentrations of 20-, 30-, 40-, 60-, and 80-percent saturation caused increased acid production in the reverse order named. In addition, he proved that high acidity resulted from the fermentations (at any given salt concentration) when cucumbers of the smaller sizes were employed. The reverse was true when the larger sizes of cucumbers were used.

Numerous investigators (10, 15, 16, 22, 26, 41) have suggested that beneficial results may be obtained by the addition of sugar to cucumber fermentations and that these benefits may be reflected in an accelerated rate of acid production, or in an increased production of lactic acid. Also, it has been reported that both acceleration and increased acid production can be obtained. However, in some cases it would seem that insufficient amount of work was done under conditions typical of the industry to justify the extensive conclusions presented. In contrast to those reporting beneficial results, Vahlteich, Haurand, and Perry (37) were unable to stimulate the acid fermentation by the addition of sugar to some of their commercial cucumber fermentations. Veldhuis and co-workers (39) were unable to demonstrate a significant increase in brine acidity of the resulting fermentations to which sugar was added to different fermentations as follows: at the start; after 10 days; and in small amounts at short intervals during the fermentation period.

YEAST FERMENTATION

Although true yeasts have been mentioned in literature in connection with cucumber fermentations Kossowicz (24); Hasbrouck (19); Riley (31); Brown (8); Le Fevre (27, 28); Joslyn (23); Vahlteich *et al.* (37); Campbell (10), no systematic study as to their populations in the brine was recorded until very recently. In 1940, Etchells (12) showed that a part of the typical fermentation of cucumbers was brought about by yeasts. Yeast fermentations were found in brine treatments of 20-, 30-, 40-, and 60-percent saturation with respect to salt. It was shown that there was no direct correlation between brine concentration and maximum number of yeasts present.

In an earlier report, Veldhuis and Etchells (38) carried out probably the first systematic study of gases evolved during the fermentation of cucumbers salted at various brine concentrations (20- to 80-percent saturation) under what could be called commercial conditions. A definite correlation was reported between carbon dioxide production and the presence of typical yeast fermentation. Another interesting point of the above-mentioned work, dealing with the presence of hydrogen in the evolved gases, will be discussed under the following topic heading.

HYDROGEN FERMENTATION

A thorough review of the literature on cucumber fermentations up until 1939 reveals no study, either chemical or bacteriological, dealing with that phase of the fermentation in which a mixture of carbon dioxide and hydrogen is evolved. However, it may be noted that hydrogen was mentioned casually upon at least two occasions when it was claimed to be a product of the undesirable microorganisms at the start of fermentation (8, 27). Upon another occasion it was claimed present among numerous other by-products (23). The first conclusive evidence that hydrogen represents a portion of the evolved gas from certain cucumber fermentations was presented by Veldhuis and Etchells (38) in 1939. In this work it was shown that hydrogen was produced in considerable quantities in all fermentations observed in 60-percent saturation brines. Examination of gas from hollow cucumbers or "bloaters" from 60-percent saturation brines showed it to have about the same composition with respect to carbon dioxide and hydrogen as the corresponding surface gas. The bacteriological investigations reported were of a limited nature. It was pointed out however, that an organism could be isolated from the typical hydrogen fermentation which was capable of producing a relatively high percentage of hydrogen.

ISOLATION STUDIES

During the 1937 season, it was found that the ordinary plating methods being used for the brine fermentations were unsuited for isolation of the causative microorganisms in brine fermentations responsible for the evolution of hydrogen. The problem of whether this difficulty was due to growth requirements of the organisms in question, or due to interference by other groups was studied somewhat in detail during the 1938 season.

It was found that in actively fermenting 40° salometer brines, where considerable amounts of hydrogen were evolved, the predominating microbial flora could consist chiefly of acid-forming bacteria and yeasts. The use of culture media suitable for the development of the latter organisms was not suited for the growth of the hydrogen-producing organisms due to limitations involving pH. When nutritive caseinate agar (Difco) was used for the

acid-formers, the pH of the medium was lowered rather rapidly by their growth. This condition was inhibitive for the hydrogen-producing organisms. The initial low pH of the medium used for cultivation of the yeasts was likewise inhibitory. Furthermore, the employment of a medium such as nutritive agar that would exclude the acid-forming bacteria and the yeasts, encouraged the spreading aerobic spore-forming types, making the plates valueless for either counting or isolation of the organisms producing hydrogen.

However, it was demonstrated that by use of strict anaerobic culture conditions,[1] a suitable differentiation of the brine microflora could be obtained on nutritive caseinate agar, and the hydrogen-producing organisms revealed in sufficient numbers so that typical colonies could be picked and studied. Once the colonial characteristics were established, numerous strains were isolated by this method.

A total of 20 strains (Table 1, part A) were isolated by anaerobic cultural methods from cucumber fermentations during the 1938 season. Of this number, eight were isolated from 40° salometer fermentations, nine from 50° salometer fermentations and three from 60° salometer fermentations. During the following season, nine additional strains were added to the collection of stock cultures (Table 1, part B). This group was divided as follows: two from 20° salometer fermentations and seven from 60° salometer fermentations. The strains isolated during 1939 were used chiefly for comparative studies, particularly in reference to gas evolution and composition.

TABLE 1—*Origin of cultures isolated from cucumber fermentations*

A. 1938 Season		B. 1939 Season	
Strain No.	Brine concentration	Strain No.	Brine concentration
	°sal.*		°sal.*
H-138	40	H-139	60
H-238	40	H-239	20
H-338	40	H-339	60
H-438	40	H-439	60
H-538	40	H-539	60
H-638	40	H-639	60
H-738	40	H-739	20
H-838	40	H-839	60
H-1138	60	H-939	60
H-1238	60		
H-1338	60		
H-1438	50		
H-1538	50		
H-1638	50		
H-1738	50		
H-1838	50		
H-1938	50		
H-2038	50		
H-2138	50		
H-2238	50		

*Percent saturation with respect to salt.

[1]Large desiccators were used for anaerobic jars, freed of oxygen by the addition of excess alkaline pyrogallol mixture followed by drawing a vacuum of 15 to 20 inches of mercury.

IDENTIFICATION STUDIES

Twenty strains (Table 1, part A) were studied in detail with respect to morphological, cultural and physiological characteristics. The procedure followed, for the most part, was according to the recommended bacteriological methods (33). When a particular method for a test or procedure different from this was used the reference is indicated by number.

During the study dealing with the cultural characteristics observed on the 10 solid and liquid media employed, complete observations with respect to the various characteristics were made for each of the 20 strains on each type of medium. This material was then analyzed and finally condensed into what might be called the typical characteristics for the type strain (H-1438) on the various media.

The combined morphological, cultural and physiological characteristics of the 20 strains are presented in the summarized discussion of results.

For completeness, additional data are included in the summary of results (such as fermentation reactions on the various carbon compounds) although this information is not fully discussed until later in subsequent sections of this report.

After establishing the purity of the cultures by successive platings and prior to planting onto the cultural media, the cultures were given two transfers; first on nutritive caseinate agar (with 8.0 cc. of 0.4-percent brom-cresol-purple per liter) and then into cucumber juice broth. From the latter, the inoculations onto the various media were made. A similar set of broth transfers was used at an age of 24 hours for the morphological, staining and motility tests.

Growth on the initial transfer stabs was best at the top, being gray, glistening and entire, with alkaline reaction extending down into the stab. Growth in the stab was out from the line of puncture and was lobate to papillate. An acid reaction prevailed in the deep part of the tube. Gas was formed in some cases in the base of the tube and in other cases was also present as small bubbles in the surface growth. When gas formed in the base of the stab, the amount was sufficient to split the agar and raise the stab to the top of the tube.

Transfers made from these stabs into cucumber juice broth showed moderate growth in 12 hours and good to abundant growth in 24 hours. At this time a delicate pellicle or film appeared. The turbidity and the amount of sediment markedly increased during the 12-hour period. Gas formation caused the broth to be heavily charged and when shaken, a foam of 2 to 3 cm. in height would rise up the tube. The broth remained turbid several days. If the pellicle was disturbed, so as to be submerged, a new one formed within 24 hours. The pellicle was glistening and sometimes flaky in appearance. Gas production was most vigorous during the first 24 to 48 hours.

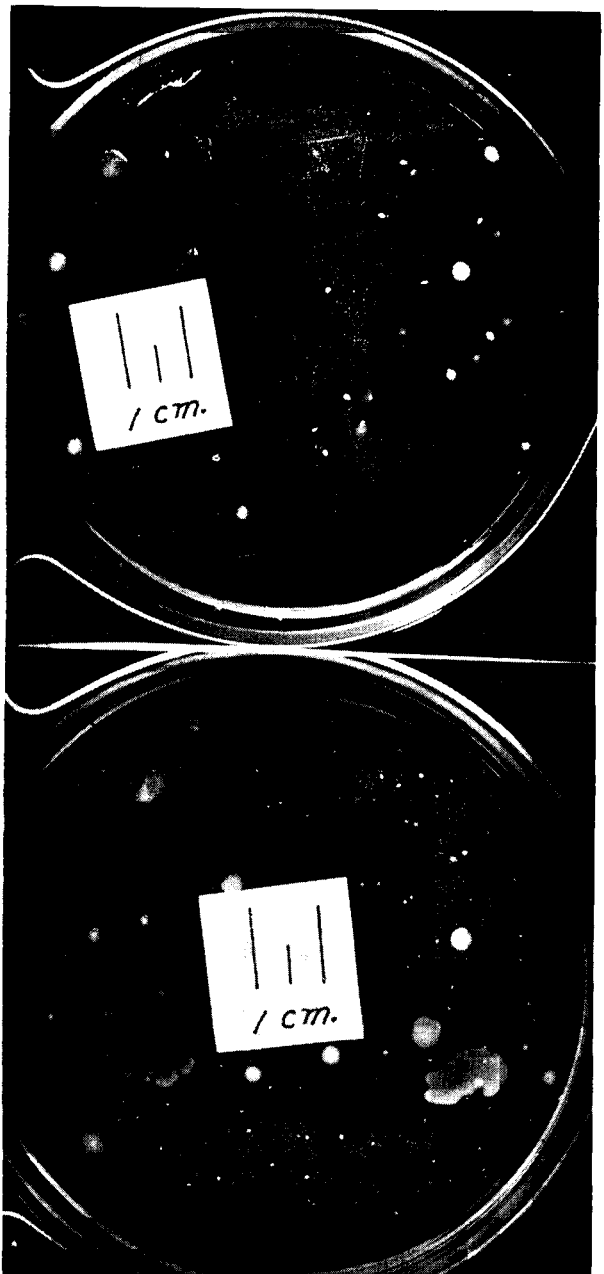

Fig. 1. Colonies of strain H-1438 after 48 hours on nutrient agar at 35° C. Note more spreading type of surface colonies and bottom growth shown on the lower plate.

Attention is called to a few of the more significant characteristics which are more or less typical of all strains. Briefly, they are as follows: (a) In nutritive caseinate agar stabs plus brom-cresol-purple, the surface growth appears to be about 0.5 square cm. in area on the top of the stab; underneath, the reaction is alkaline about one-third the way down the tube and acid in the lower part of the tube and may be accompanied by gas production. (b) In plain agar slants, there is filiform growth with typical cross-hatching or net-like structure which is irridescent and translucent in character. Also, on this medium after about one week's time, there appears a spiked, feathery growth, down into the agar at irregular intervals along the slant. (c) In cucumber juice broth, there is rapid growth with pronounced turbidity and sediment. A delicate film or pellicle follows ring formation; the broth is heavily charged with gas, rising up in a foam when shaken. Cultures remain turbid for several days and have a large amount of sediment which is viscid in character. The odor of the cultures is of a sweetish character and tends to be aromatic, resembling a yeast fermentation. After prolonged incubation on nutrient agar, the odor tends to become mildly putrefactive. (d) Growth in brom-cresol-purple milk shows slight acidity at the first reading (3 days) which increases after the one-week period and at 2 weeks there is coagulation with acid curd and evidence of trapped gas bubbles in the curd. (e) Gelatin stabs show slight to moderate growth at first, with a 0.5 square cm. area of growth at the top of the stab. Lenticular pockets are present one-third the way down the tube. Later, at two weeks, liquefaction starts from the top and the surface of the gelatin drops down in a cone shape. Marked liquefaction is found at 3 weeks. (g) Gas production in solid media containing dextrose is evidenced by the breaking up of the agar and the pushing up of the slants into the mouths of the tubes, also, by gas bubbles sometimes present directly in the surface growth on the slant.

The summarized data with respect to morphological, cultural and physiological characteristics are as follows:

MORPHOLOGICAL

Rods 0.5 x 1.0; 0.75 x 1.5; 1.0 x 2.5 microns, occurring singly, in pairs, in chains of three elements or masses and groups. Rods appear rounded on the ends and show indications of bi-polar staining. In all cases (20 strains) the majority of the cells are of the smaller size. In the masses and clumps, it seems that the cells are held together by a viscid material. They are gram-negative, non-encapsulated and motile. In old cultures (eight months) the cells appear slightly smaller although no other changes were noted and no spores were found.

CULTURAL

NUTRITIVE CASEINATE AGAR COLONIES—Growth on the surface may spread in a flat veil-like film covering the whole agar surface in the culture dish; also colonies may range from 1 to 7 cm. in diameter in fleecy, arborescent, branched filaments of growth which are irridescent and translucent. Sub-surface colonies are small, 0.5 x 1.0 x 2.0 mm. in size, lenticular in shape. However, growth on dextrose and plain agars (Fig. 1) may vary somewhat as to the colonial characteristics described above, chiefly with respect to spreading of the colonies and the branching type of growth. In general, growth on the latter media is more regular, without pronounced branched filaments.

NUTRITIVE CASEINATE AGAR SLANTS—Growth abundant, filiform to slightly spreading at base of slant, edges finely lobate to wavy, glistening, flat, smooth to slightly contoured from center line, translucent, viscid to butyrous. Odor sweet to alcoholic. Acid reaction in butt of slant and alkaline beneath surface growth. A portion of the agar broken or split in bottom due to gas production.

NUTRIENT AGAR SLANTS—Growth moderate to abundant (not so vigorous as that described for the above-mentioned medium) filiform, spreading at base of slant, glistening, flat to somewhat contoured, finely reticulated with net-like cross-hatching (some strains smooth); irridescent, translucent with streak being white to grayish and being viscid to butyrous. No color to the medium; sweet to aromatic odor; no gas detected. After one week, cross-hatching or net-like appearance shows more clearly. Plates with numerous colonies; after 6 days' incubation at room temperature a mild putrefactive odor is present; also, the odor of ammonia can be detected. After 2 weeks, the majority of the strains show a spiked to feathery growth at irregular points along the streak, extending down into the agar.

DEXTROSE-TRYPTONE AGAR SLANTS—Growth abundant, filiform to spreading, smooth edges usually entire but may be wavy. Growth glistening, liquid in appearance, surface smooth and translucent as well as transparent. Streak grayish with viscid consistency, sweetish odor, acid in base of slant and alkaline underneath growth. Gas may be present in the base of tube sufficient to raise or break the agar. After one week all strains show an alkaline reaction, in cases where the agar is broken away in the base of the tube, the lower portion shows a reduction of indicator to colorless.

CUCUMBER JUICE AGAR SLANTS—Growth abundant, filiform with slight spreading in lower portion of slant. Some strains have a wavy, transparent periphery along the edge of the streak, glistening slightly convex, but generally smooth and flat; some strains wavy to moderately contoured, translucent, irridescent, gray to ivory color, viscid to butyrous and no apparent

color given to medium; sweet to alcoholic odor. Gas production may split agar or push slant to top of tube, with bubbles being formed and trapped in surface growth. After one week some strains may develop the net-like, cross-hatched appearance described for the nutrient agar slants. After 2 weeks, growth appears more spreading in character, a secondary, arborescent growth showing from the edge of the primary streak.

CUCUMBER JUICE BROTH—Strong turbidity with delicate membrane or pellicle, which drops or disintegrates if disturbed. Moderate to abundant sediment, white, in masses, somewhat viscid and rises in a swirl. Gas present, the broth being heavily charged and if shaken a foam rises 3 to 4 cm. in height. After one week, a new pellicle is formed, and it has a glistening lustre. A perceptible sweetish odor observed. After 2 weeks a moderate clouding still exists, surface growth present, but not continuous in all strains.

NUTRIENT BROTH—Slight to moderate turbidity; membrane or delicate pellicle present in a few strains, also some with ring growth, the latter having villous projections down into the broth. The ring may fall in part into the broth. The majority of the strains are without surface growth. No odor detected. Small amount of sediment, somewhat viscid in character when present in sufficient amounts to swirl. No gas detected visually. After one week, slight to moderate persistent clouding. After 2 weeks, slight to moderate clouding, small amount of viscid sediment; no surface growth present.

POTATO SLANTS—Growth moderate to abundant, filiform, slightly irregular along the line of inoculation, glistening, smooth, slightly raised, yellowish to faint orange in varied degrees (for different strains), some appear ivory to cream in color; consistency slightly viscid to butyrous darkening the medium. Odor sweet to alcoholic. After one week, growth appears to be tan to golden. No change after 2 weeks.

BROM-CRESOL-PURPLE MILK (11)—Slightly acid in reaction, acid curd absent, rennet curd absent, peptonization absent, film of growth at surface with gas bubbles trapped underneath. After one week an increased acid reaction, slight coagulation with one strain. After 2 weeks solid curd with all strains, some extrusion of whey; pronounced acid reaction to indicator.

PLAIN GELATIN STAB—Growth filiform, finely papillate edges, gas bubbles in upper one-half to one-third of the stab along the line of inoculation; bubbles lenticular, 0.2 to 1.0 cm. in size. Liquefaction absent at 4 days. After one week the surface growth of 0.2 to 0.5 square cm. area on top of the stab is smooth, grayish and glistening; gas bubbles still visible. After 2 weeks the gas bubbles disappear and the surface growth drops, leaving a sunken area. Liquefaction of napiform character, progressing more in some strains than in others although not pronounced, being only 0.5 to 1.0 square cm. in area

and extending downward from 1.5 to 3.0 cm. After 3 weeks there is considerable liquefaction being complete in two strains, almost complete in 12 strains, and about one-half complete in the other six strains; infundibuliform to stratiform in character.

PHYSIOLOGICAL

INDOLE (7)—Not produced (by all strains).

NITRATES (40)—Reduced (by all strains).

HYDROGEN SULFIDE (42)—Not produced (by all strains).

CATALASE (17)—Produced (all strains).

METHYL RED TEST—Negative with majority of strains, a few doubtful, two (H-138 and 238) positive.

VOGES-PROSKAUER TEST—Positive (all strains).

URIC ACID TEST (2)—Uric acid can be utilized as the sole source of nitrogen by most strains. H-138 and 238 doubtful.

CITRIC ACID TEST (2)—Citric acid can be utilized as the sole source of carbon by all strains.

EFFECT OF ORGANIC ACIDS—Growth usually inhibited by about 0.05-percent acetic acid and 0.1-percent lactic acid.

CLEAVAGE OF CARBON COMPOUNDS—Demonstration of cleavage by evolution of gas from: l-arabinose, dextrose, d-galactose, lactose, levulose, maltose, d-mannose, d-mannitol, raffinose, rhamnose, saccharose, salicin, d-sorbitol, and l-xylose; cleavage was demonstrated for the following additional compounds by the indicated number of strains: dextrin, 3; glycerol, 14; inulin, 3; starch, 11; lemon pectin, 2. Melezitose was not attacked by any of the strains. Likewise, cellulose was not attacked by the strains tested.

RELATION TO OXYGEN—Aerobic, facultative, will grow abundantly under both aerobic and anaerobic conditions. Anaerobic growth demonstrated by cultivation in solid and liquid media in an anaerobic jar, (large desiccator).

RELATION TO SALT—Growth in concentrations upwards of 80° salometer (21.2-percent salt) in normal cucumber fermentations. However, under ordinary laboratory conditions, using liquid media, growth is not observed visually in salt concentrations exceeding about one-half the above amount. Some strains may be made to increase in salt tolerance when the sub-cultured serially in liquid media containing salt. Salt is not necessary in ordinary media for abundant growth.

THERMAL DEATH TIME—Instantaneous to 6 minutes exposure at 60° C., depending on the strain. Cultures able to withstand 50° C. for 10 minutes.

OPTIMUM GROWTH TEMPERATURE—30 to 35° C. No gas produced at 45° C., although growth may be noted.

OPTIMUM pH CONDITIONS—Maximum gas production in a buffered series, pH 5.1 to 5.3. Excellent gas production occurred in unbuffered dextrose broth, equal to or exceeding that of the buffered lot.

The foregoing data with respect to morphological, cultural and physiological characteristics (as well as the biochemical studies on acid production and end-products of the fermentation which are discussed shortly) assure positive identification of the 20 cultures as belonging in the genus *Aerobacter* (Beijerinck) described in Bergey's manual (6). Only two true species of the genus are recognized in that manual; they are *Aerobacter aerogenes* and *Aerobacter cloacae*. Both species have a great number of characteristics in common; however, they differ chiefly with regard to fermentation of glycerol and liquefaction of gelatin. With *A. aerogenes* glycerol is fermented and gelatin is not liquefied while with *A. cloacae* the reverse is true for the action on both compounds.

TABLE 2—*Acid production from the fermentation of dextrose broth after 4 days incubation at 35° C.*

MEDIUM: 0.5-percent dextrose, 0.5-percent K_2HPO_4, 0.5 percent each of tryptone and peptone.

METHODS: Two cc. aliquots diluted with 35 cc. of distilled water, brought to a boil to expel CO_2, cooled and titrated with 0.111 N NaOH, using phenolphthalein as the indicator; values obtained expressed as grams lactic per 100 cc. of medium. pH determinations by the glass electrode.

Culture No.	Final pH	Grams lactic acid per 100 cc.		Culture No.	Final pH	Grams lactic acid per 100 cc.	
		I	C			I	C
Broth control	7.38	0.120	.....	H-1738	7.22	0.120	0.000
H-138	6.38	.300	0.180	H-1838	7.10	.130	.010
H-238	5.92	.360	.240	H-1938	7.25	.080	−.040
H-338	7.60	.070	−.050	H-2038	7.32	.100	−.020
H-438	7.52	.070	−.050	H-2138	7.35	.120	.000
H-538	7.52	.090	−.030	H-2238*	7.02	.130	.010
H-638	7.40	.080	−.040	H-139	7.02	.165	.045
H-738	7.38	.080	−.040	H-239	7.48	.070	−.050
H-838	7.38	.080	−.040	H-339	6.85	.180	.060
H-1138	6.92	.140	−.020	H-439	7.36	.100	−.020
H-1238	6.96	.125	.005	H-539	7.02	.150	.030
H-1338	7.58	.070	−.050	H-639	7.12	.130	.010
H-1438	7.28	.120	.000	H-739	7.58	.070	−.050
H-1538	7.45	.150	.030	H-839	7.08	.150	.030
H-1638	7.48	.100	−.020	H-939	7.46	.075	−.045

I: Values include acid of broth control.
C: Corrected values.
* Cultures through H-2238 from 1938 series, remaining cultures from 1939 series.

Taking into account the above-mentioned reactions as well as other characteristics of the group as a whole, 18 of the 20 cultures can be considered allied to the species *cloacae* although the characteristics are by no means identical. Within the group are several cultures that by virtue of their action on glycerol and starch, as well as other characteristics, may be considered as intermediates and are allied to both the species of the genus *Aerobacter, namely, A. aerogenes* and *A. cloacae*. The remaining two cultures (H-138 and H-238) must be considered as further varieties of *A. cloacae*.

ACID PRODUCTION

When viewed in the light of known behavior of the *Aerobacter,* the results of the following investigation upon acid production from various compounds by all strains becomes more understandable.

One-percent solutions of various carbon compounds* were titrated at the conclusion of a 2-week incubation period and the values with respect to amounts of N/10 acid or alkali required for neutralization showed that in the majority of the cases, the final reaction to the indicator was alkaline, necessitating titration with acid. Of 20 strains tested only two, H-138 and H-238 (varients), showed any consistency in fermenting the compounds with resulting acid production which was still present at the time of analyses. In general, it would appear that for the majority of the strains tested, the final acidity after 2 weeks' incubation was negligible or absent. The results of a subsequent investigation, dealing with the acid production from dextrose after 4 days' incubation (Table 2), serve to confirm the preceding data. In the case of the fermentation of dextrose, out of 29 cultures studied, 14 (or 48 percent) showed negative values after correcting for the control. The rest of the cultures (with the exception of H-138 and H-238) were either neutral or nearly so.

If appreciable amounts of acid were produced by the *Aerobacter* cultures, other than the exception noted, it was either neutralized by alkaline compounds formed or it was destroyed to form other compounds. Previous work points toward the latter condition being brought about. The reversion of acidity caused by the *Aerobacter* has been shown by Ayers and Rupp (5). Also, these workers pointed out that this reversion could be accomplished under conditions which were independent of alkali production. Furthermore, they demonstrated that the acetic acid formed by the organisms during the dextrose fermentation was in part destroyed. The findings of Reynolds and Werkman (30) confirmed those of Ayers and Rupp and in addition showed that the reversion of the accumulated acetic acid was accompanied by an increase in acetylmethylcarbinol and 2, 3-butylene glycol brought about by condensation and reduction.

*l-arabinose, dextrose, dextrin, glycerol, d-galactose inulin, lactose, levulose, maltose, d-mannose, d-mannitol, melezitose, raffinose, rhamnose, saccharose, salicin, d-sorbitol, starch, l-xylose in basal broth of 0.5 percent tryptone and 0.2 percent yeast extract.

END-PRODUCTS OF THE FERMENTATION

It has been shown (18, 32) that the fermentation of dextrose by *Aerobacter aerogenes* yielded formic, lactic and succinic acids, as well as carbon dioxide, hydrogen, ethyl alcohol, 2, 3-butylene glycol, and acetylmethylcarbinol. Of these products, it has been suggested (32) that succinic acid probably resulted from the protein present in the culture medium. In an investigation of the dissimilation products of dextrose by *Aerobacter indologenes* (regarded by the authors of Bergey's manual as a variety of *A. cloacae),* Reynolds and Werkman (30) showed that the following products were formed: formic, acetic and lactic acids, ethyl alcohol, hydrogen, carbon dioxide, acetylmethylcarbinol and 2, 3-butylene glycol. Of these products, formic and acetic acids and acetylmethylcarbinol decreased in amount following prior accumulation. The formic acid was decomposed to carbon dioxide and hydrogen; the acetic acid to acetylmethylcarbinol and then the latter to 2, 3-butylene glycol.

It is evident from the foregoing discussion that the products from the fermentation of dextrose by both species of the genus *Aerobacter* are essentially the same.

In the present studies, an investigation of a limited nature upon the fermentation of dextrose by type strain H-1438 (Table 3) showed the following products were formed; carbon dioxide, hydrogen, ethyl alcohol and esters (trace). Also, the fermented liquor gave a strong test (qualitative) for the

TABLE 3—*Fermentation end-products by Strains H-1438 (type strain) and H-138 (variant) from dextrose broth after 4 days' incubation at 35° C.*

MEDIUM: Fermentations were carried out in one liter flasks containing 400 cc. of medium with the following ingredients per 100 cc; 1.0 g. of dextrose, 0.5 g. of K_2HPO_4, 0.2 g. of yeast extract, 0.5 g. of tryptone.

METHODS: Titratable acidity by the method described in Table 2. Volatile acids and esters according to the A.O.A.C. (3); Ethyl and butyl alcohols by the method of Stahly, Osborn, and Werkman (34); Gases evolved collected over saturated acidified brine solution and analyzed according to method described by Veldhuis and Etchells (38); pH determinations by the glass electrode.

Strain	Products in grams per 100 cc. of medium						
	Titratable acidity as lactic	Volatile acids as acetic	Alcohols		Esters as ethyl acetate	Gases	
			ethyl	butyl		CO_2	H_2
H-1438	−0.065*	a	0.113	0	0.03**	0.388	0.007
H-138	0.268*	0.027	0.073	0	0.02	0.159	0.004

*Values corrected for acid (0.102 g./100) present initially in the medium.

**Values corrected for blank (0.005 g./100) run on the medium.

a Absent, distillate alkaline.

N.B. Initial pH of the medium was 7.18; after 4 days of fermentation by strains H-1438 and H-138 the values were 7.68 and 5.38 respectively.

presence of acetylmethylcarbinol. The determination for butyl alcohol was negative. The fermentation by strain H-138 (variant) resulted in the production of non-volatile acids (probably, chiefly lactic) and volatile acids in addition to the products mentioned for strain H-1438.

In view of the earlier discussion of the destruction of acetic and formic acids by the *Aerobacter,* it is probable that this behavior accounts for the absence of acids in the case of the 4-day fermentation by strain H-1438. If such is to be presumed, then it is not at all unlikely that little or no lactic acid was produced, since reversion of the acidity was complete. However, with the fermentation by the variant (H-138) the above-mentioned relationship with regard to reversion of acidity was not pronounced since an appreciable amount of non-volatile acid as well as a determinable amount of volatile acid was found at the conclusion of the 4-day fermentation period.

PRELIMINARY BIOCHEMICAL STUDIES

In the present investigation, a study of the amount and composition of gases evolved from fermentation has been made by employing quantitative methods of analysis. Fermentations resulting from media containing various carbon sources, such as carbohydrates, alcohols and glucosides have been examined with respect to the components of the gases evolved. During the fermentation of dextrose, several of the factors influencing the fermentation proper have been investigated. This constitutes the principal work involved although other investigational phases are included.

PRELIMINARY EXPERIMENTS

A considerable amount of preliminary work was carried out relative to the preparation and methods of handling of a suitable apparatus for gas collection, since specific requirements were necessary. In addition, several preliminary fermentations testing the apparatus were conducted.

A drawing giving the specifications of the gas collection apparatus designed for the studies is shown in Fig. 2. Prior to using, it was sterilized at 15 pounds pressure for 10 minutes with an empty flask attached to protect the lower part of U-tube F. Prior to autoclaving, a small cotton plug was placed well up into the U-tube F that separates the culture medium from the collection flask A. The sterile apparatus was handled as follows:

A sterile 50-cc. round-bottomed flask containing the culture medium was inoculated and substituted for the empty flask. Saturated, acidified brine solution was placed in flask B and was brought up to the bottom of the rubber stopper in flask A through U-tube E and up into the gas outlet tube D by suction on a short piece of rubber tubing attached to D. The gas outlet was then sealed with a screw clamp. Incubation was at 35° C. One of the

chief advantages of this type of apparatus, other than its simple construction and ease of handling, is that because of its small size and compactness several individual fermentations can be carried out at the same time in a limited amount of incubation space.

The procedure in handling was as follows: The solutions to be tested were transferred to 125-cc. flasks, heated to 80° C. in a water bath, then sterilized by autoclaving at 10 pounds pressure for 10 minutes. Where compounds were used that were susceptible to heat, sterilization was carried out by Seitz filtration. The sterilized solutions were then poured aseptically to the graduated mark on the neck of the sterile 50 cc. calibrated culture flasks. After inoculation the flasks were placed on the gas collection outfits. Saturated, acidified brine solution was added to the receiving flasks and after the outfits were observed for one hour for leakage they were placed in the incubator.

METHODS OF ANALYSES

The gas was measured and analyzed in a modified Williams gas analysis outfit according to a procedure previously described (38). Tests for methane or other combustible hydrocarbons were made by passing the products from the hydrogen determination through potassium hydroxide and noting the decrease in volume. This test was always negative and is not reported. Whenever possible, a 100-cc. volume of gas was analyzed as the apparatus was designed for maximum efficiency at this volume. Analyses of small volumes of gas (10 to 20 cc.) introduced a considerable error, brought about principally by: (a) insufficient amount of gas available so that a portion could be used for washing out the manifold and (b) difficulty in manipulation of the small amount of gas through the absorption solutions so as to obtain adequate contact. Residual (dissolved) gas remaining in the medium was determined by boiling the contents of the culture flask and trapping the gas driven off. In cases where media were buffered at alkaline pH's the culture flasks after fermentation were first acidified, then boiled to free the carbonates as carbon dioxide. In some instances this gas was analyzed separately, while in others it was incorporated in the collected gas and the total gas analyzed. This method of determining the amount of residual gas was not wholly accurate since a small amount of gas was directly above the liquid; however, it gave a reasonable indication of the volume of the dissolved gas. Also, the analyses of the residual gas as to components are not comparable in accuracy to gas analyses run on larger volumes.

It should be noted at this time that in the gas analyses, the oxygen values were always found to be below the amount known to have been initially present at the start of fermentation. By this is meant, that if the oxygen value were multiplied by 4 to give the estimated nitrogen, and the sum of the two volumes were considered to represent the volume of air, that result did not

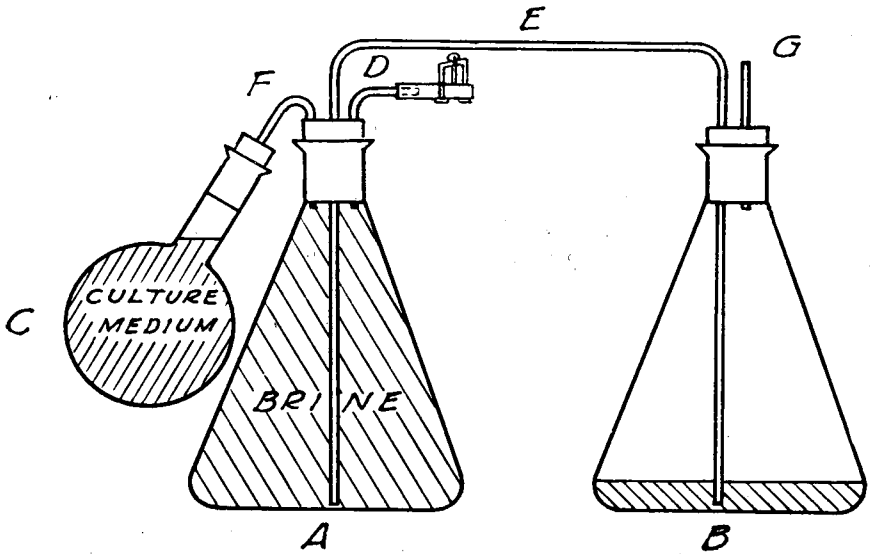

Fig. 2. The apparatus consists of one 50-cc. and two 250-cc. flasks. Flask A fitted with a three-hole, No. 5 rubber stopper which is supplied with a short right-angle tube D for gas outlet; a short U-tube F for attaching the 50-cc. round bottom culture flask C by means of a No. 0 rubber stopper; a long U-tube E for connecting flasks A and B. Flask B fitted with a two hole No. 5 rubber stopper through which passes the U-tube from flask A and an air outlet tube G. A short piece of rubber tubing is attached to tube D and supplied with a screw clamp when fermentation is started.

equal the amount of air present above the culture medium at the start of fermentation. This would indicate that some of the oxygen was utilized by the fermentation as well as possibly being dissolved in the liquid system. This condition, especially in analyses of gas from active fermentations, favored a lower value of gas accounted for in the analyses since the remaining gas was calculated as nitrogen and this figure was calculated from the oxygen found present. Usually, the amount of gas accounted for by analysis ranged from 95-99 percent of the total measured.

During the fermentation period the brine level in the gas receiving flask A (Fig. 2) was marked each day and at the conclusion of the experiment the rate of gas production was determined by the amount of brine displaced each day.

RESULTS

The result of the fermentations of cucumber juice broth as to gas evolution and composition show that this medium yielded gas composed of 81.0 percent carbon dioxide and 16.4 percent hydrogen, based on the mean values of five determinations. The ratio of hydrogen to carbon dioxide was 1 : 5. There were variations between individual fermentations although not suf-

ficient to be considered significant. The analyses of the residual gas showed that it possessed a composition essentially the same as that which was evolved.

The fermentations of dextrose broth with respect to gas production and composition showed that the percentages of carbon dioxide and hydrogen were similar regardless of the initial pH adjustment. No significant differences were noted that were not exceeded by variations within the duplicates. The ratio of hydrogen to carbon dioxide for the three lots at pH 5.05, 6.80 and 9.05 were: 1 : 2.1; 1 : 2.3 and 1 : 2.3 respectively. There is practically no difference between any two of the three ratios. However, they do show a considerable increase in proportion of hydrogen when compared with cucumber juice broth fermentation which was 1 : 5.

The gas production for the above-mentioned series was recorded at intervals up to 14 days and the results are presented graphically in Fig. 3. It will be seen that the fermentation in all lots was rapid, with little gas being produced after 3 to 4 days' incubation. The fermentation in the unadjusted lot (pH 6.8) was the most rapid and showed some increase in gas production when compared with the other lots adjusted to pH 5.05 and 9.05.

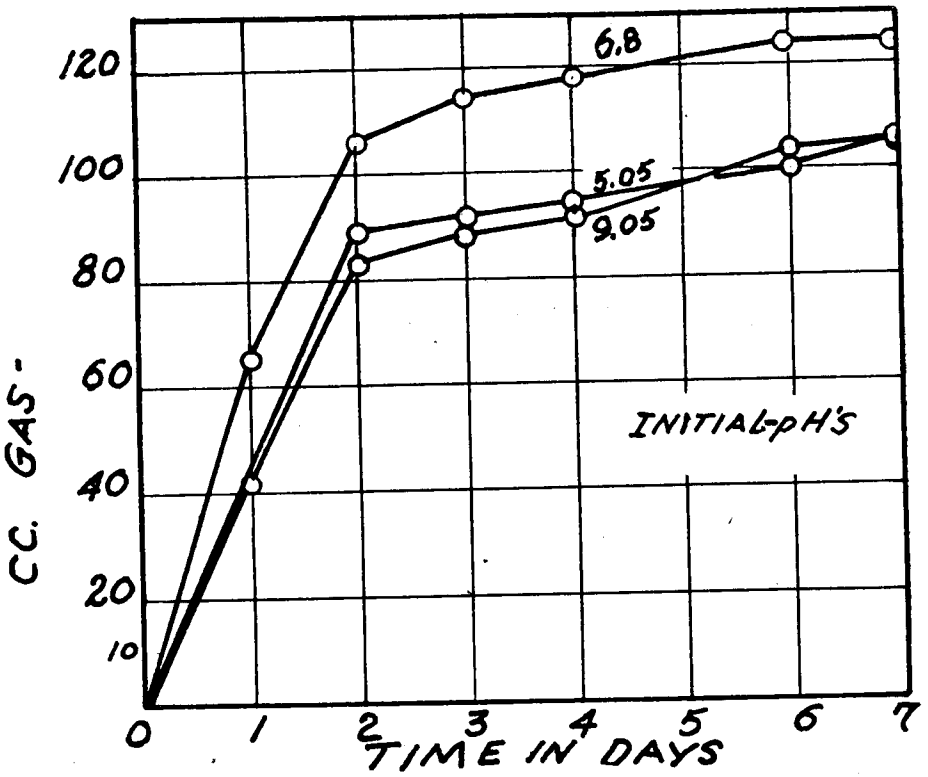

Fig. 3. Effect of initial pH adjustment of the culture medium upon gas evolution from the fermentation of dextrose.

The most important results to be noted from these preliminary experiments were: (a) a satisfactory apparatus for studying individual fermentations was devised and tested; (b) the gases produced from cucumber juice and dextrose proved to be composed solely of hydrogen and carbon dioxide and that the fermentations varied, depending upon the carbon source fermented; (c) the fermentations occur over a considerable range with respect to initial pH adjustment, and (d) during fermentation the gas production was rapid, the major portion being produced within about 48 to 72 hours.

PRINCIPAL BIOCHEMICAL STUDIES

The preliminary investigations were followed with more extensive fermentation studies with respect to gas production, rate of gas production and composition of the evolved gases. More specifically, the experimental work, in relation to the foregoing general grouping, covered the following phases: (A) fermentations by different strains; (B) fermentations by the same strain; the effect of (C) temperature, (D) pH and, (E) salt* upon the fermentation of dextrose; (F) the fermentation of various carbon compounds.

PROCEDURE

The gas collection outfits, methods of gas analyses, determination of residual gas, determination of the rate of gas evolution, and the preparation of the test carbon compounds have all been previously described.

General procedure not previously described will be presented in part at this time; the rest will be given when the individual experiments under consideration are presented.

The basal broth to which the fermentable carbon compounds were added was modified slightly (from the original description) to exclude yeast extract, since this material had a tendency to promote frothing in the neck of the culture flasks during fermentation. The final basal broth consisted solely of tryptone (0.5 percent), which was found satisfactory for the growth requirements of the organisms.

For the fermentations in buffered broth, the procedure was as follows: A stock buffer solution was prepared containing 20 g. of ammonium dihydrogen phosphate, 45 g. of dipotassium hydrogen phosphate and 71 g. of citric acid per liter. One hundred-cc. quantities of this buffer solution were adjusted to the desired pH values with 10 N sodium hydroxide. Ten cc. of the adjusted stock solutions per 100 cc. of basal medium was usually sufficient to obtain the required buffering effect. In experiments involving the use of a liter or more of culture medium, it was found practicable to use one-tenth of the amounts of the chemicals listed above in one liter of the basal broth

*The effect of salt upon the fermentation of cucumber juice is also included.

and then adjust this amount with 1 N sodium hydroxide to the desired pH. This procedure was used to obtain the buffered broth (pH 5.15) used in a number of the experiments.

All gas volumes reported were calculated on the basis of gas resulting from 50 cc. of culture medium containing 1.0 percent of the carbon compounds,* which would be in terms 0.5 g. of fermentable carbon source present.** Actually, the fermentation culture flasks held from 55.5 to 58.0 cc.; hence, such a calculation is in keeping with the actual amount of fermentable carbon source tested. The hydrogen and carbon dioxide ratios, in all cases, are based on the actual volumes of these gases produced and include both evolved gas (also designated as collected gas) and residual gas.

In the major portion of the experiments, the gas analyzed represented an aliquot from the total volume of a given fermentation and included the residual gas incorporated at the time of analyses. However, in two sets of experiments (D and F) residual gas was determined and analyzed separately.

All fermentations, with the exception of the temperature series (C), were conducted at 35° C. Gas volumes were corrected to room temperature (23° C. for these experiments). Corrections as to variations in barometric pressure were too small when applied to 100-cc. gas volumes to be considered significant. All inoculations were made from actively growing, 24- to 48-hour cultures, inoculation being made with one drop of culture added by a sterile pipette. Strain H-1438 was used in all experiments as the test organism with the exception of the comparative study upon fermentations brought about by several strains (A). All pH determinations were made with a glass electrode.

RESULTS

A. *Fermentations by Different Strains*

Several strains were investigated with respect to production and composition of gas. The fermentations were carried out in buffered (pH 5.15) dextrose (1.0 percent) broth containing 0.5-percent tryptone. The following strains were used: H-739, H-439, H-639 (1939), H-638, H-1338, H-438 and H-138 (1938 series). Incubation was for one week at 35° C. The gas outfits were marked at 24-hour intervals for rate of gas production (Fig. 4). At the conclusion of the incubation period the gas in the different outfits was analyzed.

The detailed results are presented in Table 4. The composition of gas from the fermentations with the various strains was the same except for strain H-138. The gas from six strains showed hydrogen to carbon dioxide ratios between 1 : 2.32 and 1 : 2.59. The maximum amount of gas produced

*With the exception of lactose, raffinose, rhamnose and salicin, here the amount was increased to 3.0 percent to obtain sufficient gas for analysis.

**Where cucumber juice was employed as the nutrient medium, the calculations were based on gas production from 50 cc., containing 2.0 percent reducing sugars (referred to as 1.0 g. of CHO present).

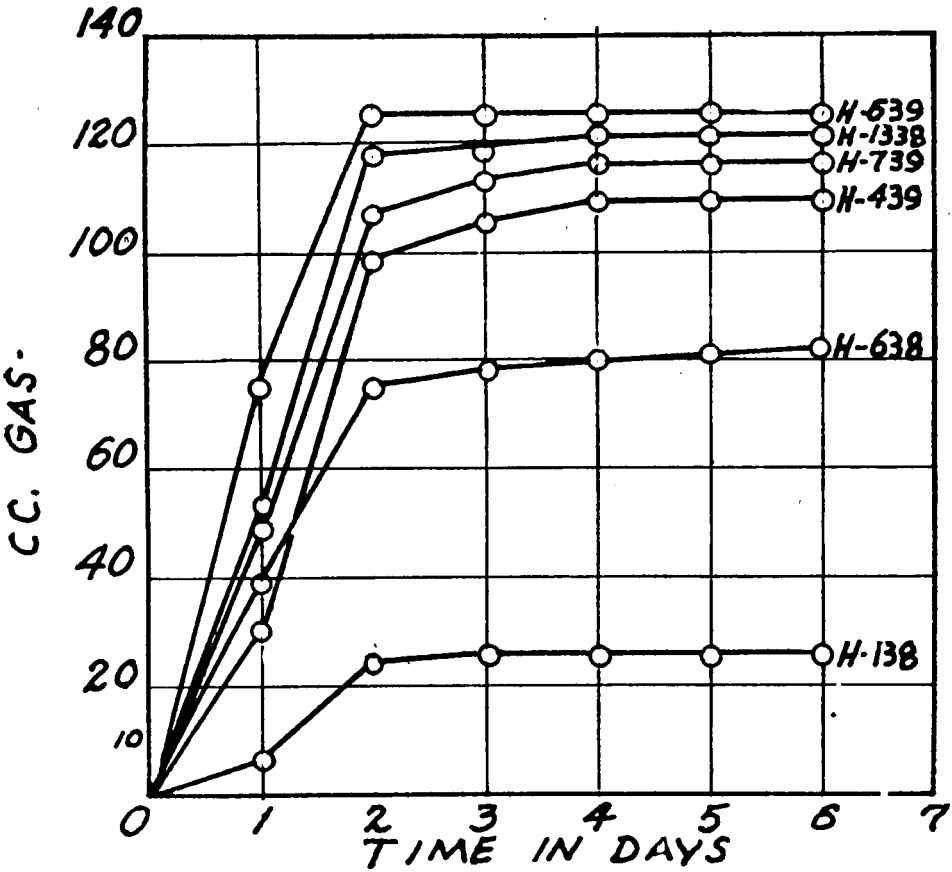

Fig. 4. Gas evolution from the fermentation of dextrose by several strains of the stock culture collection.

by five of the foregoing group also was similar, the range being from about 139 to 148 cc. However, in the case of strain H-138 a difference in behavior was demonstrated, not only by a decrease in the amount of gas produced but also by the proportion of hydrogen in the gas which was greater (1 : 1.44).

In general, it is noted (Fig. 4) that the fermentation with all strains is rapid, with the major portion of the gas evolved within 48 hours. In these fermentations, little or no gas was produced after four days. Also, the gas evolution curves show that five* of the seven strains tested produced gas volumes well above the 100-cc. range.

*Curve for H-438 inadvertently omitted; data indicate it would be practically identical with H-1338.

TABLE 4—*Fermentation of dextrose broth, buffered at pH 5.15, by several strains of stock culture collection*

Strain	Outfit No.	Total volume of gas*	Carbon dioxide		Hydrogen		Ratio of $H_2 : CO_2$ **	O_2	Remainder gas†	Residual gas	Gas accounted for by analysis
		cc.	%	cc.	%	cc.		cc.	cc.	cc.	%
H-739	1	139.3	69.0	96.1	27.9	38.9	1:2.47	.3	4.0	13.3	97.9
H-439	2	129.0	68.8	88.8	29.6	38.2	1:2.32	.3	1.7	12.3	99.4
H-639	3	145.7	70.0	102.0	28.0	40.8	1:2.50	.6	2.3	15.6	100.0
H-638	4	102.5	70.3	72.1	27.4	28.1	1:2.56	.3	2.0	12.3	99.2
H-1338	5	146.7	71.0	104.2	27.3	40.1	1:2.59	.3	2.1	17.0	99.3
H-438	6	140.0	70.8	99.1	27.3	38.2	1:2.59	.3	2.4	13.8	99.1
H-138	7	33.3	55.7	18.5	38.5	12.8	1:1.44	.3	1.7	4.4	99.2

* Values based on fermentation of 50 cc. of 1 percent dextrose broth; gas volumes include residual gas.

** Calculated from volumes of H_2 and CO_2.

† Principally nitrogen, also includes experimental error.

B. *Fermentations by the Same Strain*

The results of quadruplicate fermentations of dextrose broth by strain H-1438 are shown in Fig. 5.

The percentages of carbon dioxide and hydrogen are comparable for all fermentations, the greatest variation observed being about two percent for each component. When calculated on the basis of hydrogen to carbon dioxide ratios the range was from 1 : 2.35 to 1 : 2.49, such differences being considered insignificant. The detailed results are shown in Table 5.

C. *The Effect of Temperature on the Fermentation of Dextrose*

The influence of temperature of incubation with respect to gas production is shown in Fig. 6. Eight temperatures, ranging from 5° C. to 45° C. were employed. Experimental procedure concerning the broth, inoculation

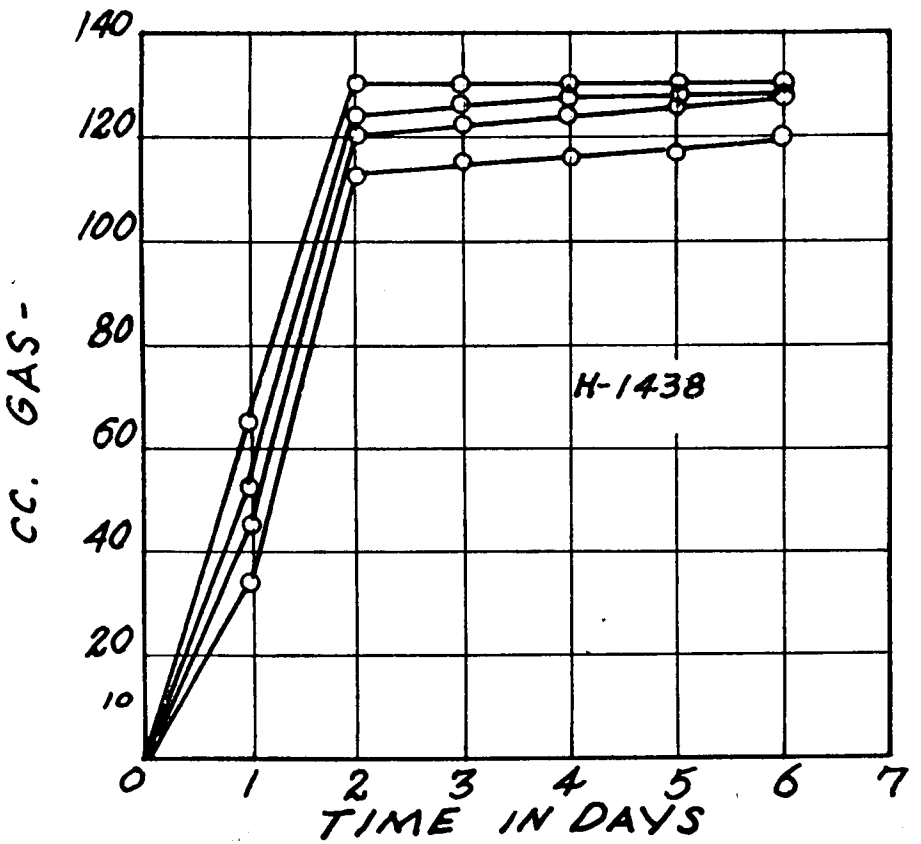

Fig. 5. Gas evolution from quadruplicate fermentation of dextrose by strain H-1438

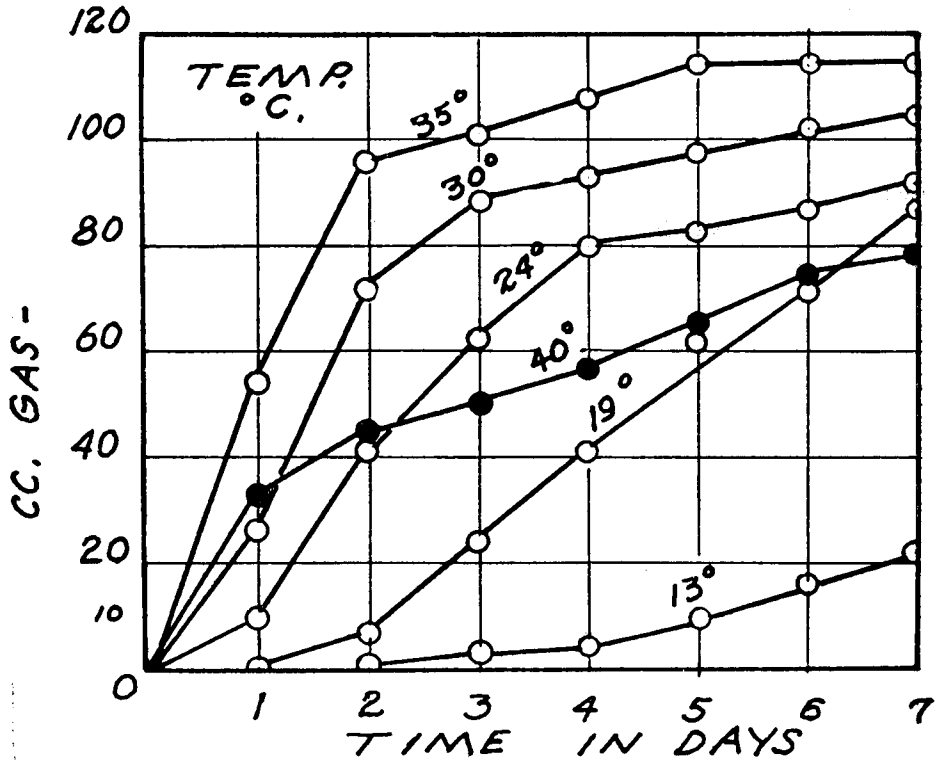

Fig. 6. Effect of temperature on gas evolution from the fermentation of dextrose by strain H-1438.

and the strain used have been previously described. The lower and higher limitations for the fermentation yielding gas were found to be 5° C. and 45° C. No perceptible growth was noted at the lower limit, while at the higher limit, perceptible growth was observed but no gas detected, either evolved or residual. At 13° C. almost one-half of the produced gas was residual, owing to the greater solubility of carbon dioxide at lower temperature. The temperatures 19°, 24° and 40° C. were similar with respect to the total amount of gas present at the end of fermentation. It would seem that the optimum temperature for gas production lies within a range near 35° C. The composition of the gas from all fermentations (13° to 40° C.) was comparable as to percentages of hydrogen and carbon dioxide found: H_2 range; 28.1 to 33.5 percent and CO_2 range; 64.6 to 69.2 percent.

The effect of temperature on the gas evolved and rate of evolution is shown in Fig. 6. The most rapid evolution, as well as the maximum amount of gas evolved, was at 35° C. Fermentations at temperatures either above or somewhat below the optimum (35° C.), namely at 40° and 24° C., were

TABLE 5—*Comparison of quadruplicate fermentations of dextrose broth, buffered at pH 5.15, by Strain H-1438*

A. Gas Composition

H-1438	Total volume of gas*	Carbon dioxide		Hydrogen		Ratio** of $H_2 : CO_2$	O_2	Remainder gas†	Residual gas	Gas accounted for by analysis
	cc.	%	cc.	%	cc.		cc.	cc.	cc.	%
1	132.7	68.6	92.4	29.3	37.6	1:2.45	.5	2.2	11.4	99.9
2	149.5	70.0	104.7	28.5	42.6	1:2.45	.3	1.9	17.0	99.5
3	148.6	70.2	104.3	28.1	41.8	1:2.49	.3	2.3	17.2	99.3
4	143.0	69.0	98.7	29.4	42.0	1:2.35	.3	1.8	15.0	99.9

* Values based on fermentation of 50 cc. of one percent dextrose broth; gas volumes include residual gas.

** Calculated from volumes of H_2 and CO_2.

† Principally N_2, also includes experimental error.

considerably retarded and less gas was evolved. Gas evolution at 19° C. was much slower than at 24° C., but at the end of the incubation period (8 days) the amount of gas evolved was about the same. At 13° C., the gas produced during the first 3 to 4 days was dissolved in the culture medium. It is evident that this temperature definitely retarded the fermentation.

D. *The Effect of pH on the Fermentation of Dextrose*

For this series, 100-cc. amounts of the stock buffer solution, prepared as previously described, were adjusted with 10 N sodium hydroxide to cover a range of eight pH values. Ten-cc. amounts at each desired range were supplemented with 1.0 g. of dextrose and 0.5 g. of tryptone and made up to 100-cc. volume. Determinations with respect to pH were made before and after sterilizing. The final pH values after sterilization for the different lots extended from 3.6 to 8.85. A control without added buffer (pH 6.8) was included.

The results show (Fig. 7) that the fermentation occurs over a considerable pH range. No growth resulted at pH 3.6 in the acid range while pH

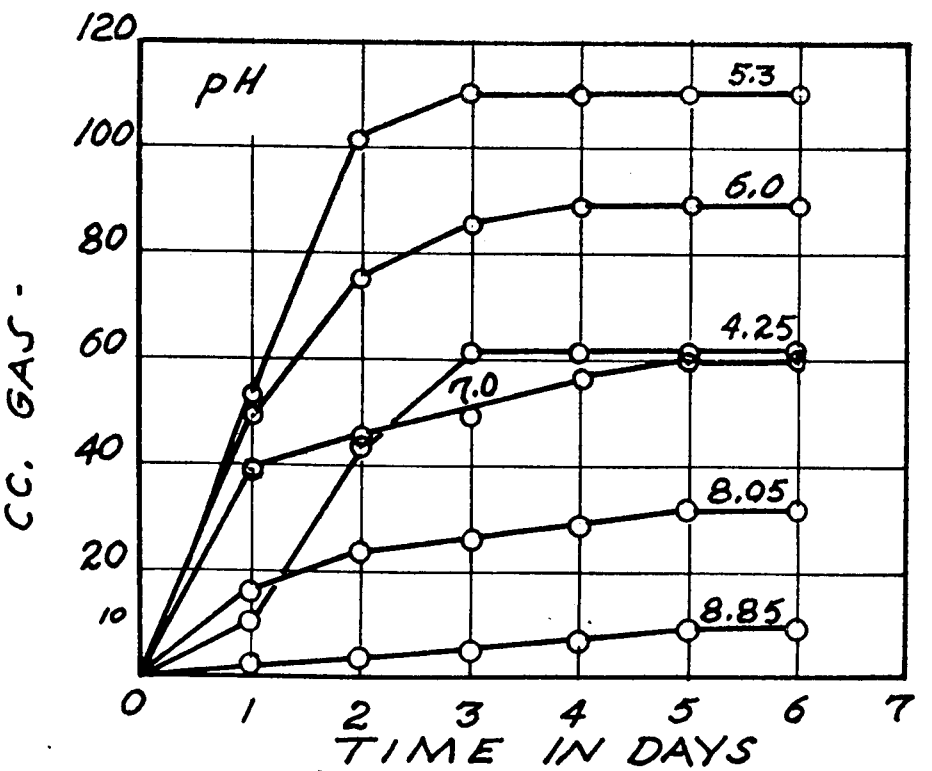

Fig. 7. Effect of buffered pH on gas evolution from the fermentation of dextrose by strain H-1438.

8.85 seemed to approach the limit for adequate growth in the alkaline range; the latter being assumed on the basis of marked decrease in gas evolution at this pH. Of the pH values studied, 5.3 seems to be the optimum in the buffered series, although the unbuffered control (initial pH 6.8) resulted in a slightly greater volume of total gas produced. The H_2 to CO_2 ratios for the series were as follows: pH 4.25, 1 : 1.71; pH 5.3, 1 : 2.15; pH 6.0, 1 : 2.29; pH 7.0, 1 : 2.07; pH 7.55, 1 : 1.76; pH 8.05, 1 : 1.65; pH 8.85, 1 : 1.64; unbuffered control pH 6.8, 1 : 2.36. In general, considering the hydrogen and carbon dioxide ratios, it is shown that no great difference in gas composition existed, although there seems to be a slight increase in proportion of hydrogen from the fermentations where the pH range increases above 7.0; likewise, this seems probable for the lower value, namely, 4.25.

The analyses of the residual gas from the fermentations showed that the amount of residual gas increased with the more alkaline pH values (9.8 cc. for pH 4.25 to 16.6 cc. for pH 8.05), owing to the reaction of the carbon dioxide with alkali.* At pH 8.85 a total of only 21.6 cc. of gas was obtained; of this amount, 7.6 cc. was released from the culture medium by acidification and boiling. The percentage of carbon dioxide was found to be about the same in all cases (range, 77 to 80 percent), and this gas constituted the major portion of the total residual gas.

The rate of gas evolution for the buffered series is presented (with the exception of that at pH 7.55**) in Fig. 7. A comparison of the gas evolution curves shows that the most rapid evolution of gas, as well as the greatest amount, resulted from the fermentation at pH 5.3. At pH 6.0 the rate of gas evolution was not particularly influenced, although there was a decrease in the amount of gas evolved. Fermentations at pH 4.25, 7.55 (not shown) and 7.0 were comparable with respect to rate of gas evolution, the major portion of the gas being evolved within 3 to 4 days. However, only slightly more than one-half of the amount of gas was evolved from these fermentations as compared to the optimum (pH 5.3). The effect of the more alkaline pH values (8.05 and 8.85) was shown most clearly by a marked decrease in gas evolution. The same relationship was true for the total amount of gas produced (evolved plus residual).

E. *The Effect of Salt (NaCl) on the Fermentation of Dextrose and Cucumber Juice*

Calculated amounts of C. P. salt were added to 100 cc. volumetric flasks and made up to volume with 1.0-percent dextrose broth buffered at pH 5.15 with the stock buffer mentioned previously. The salt concentrations employed were: 5-, 10-, 15-, 20- and 25-percent saturation with respect to salt. This series, after sterilization, was inoculated with strain H-1438 and

*Lots fermented at pH values above 7.0 were acidified prior to boiling to release the carbon dioxide.
**Omitted for clarity in presentation; followed 7.0 very closely.

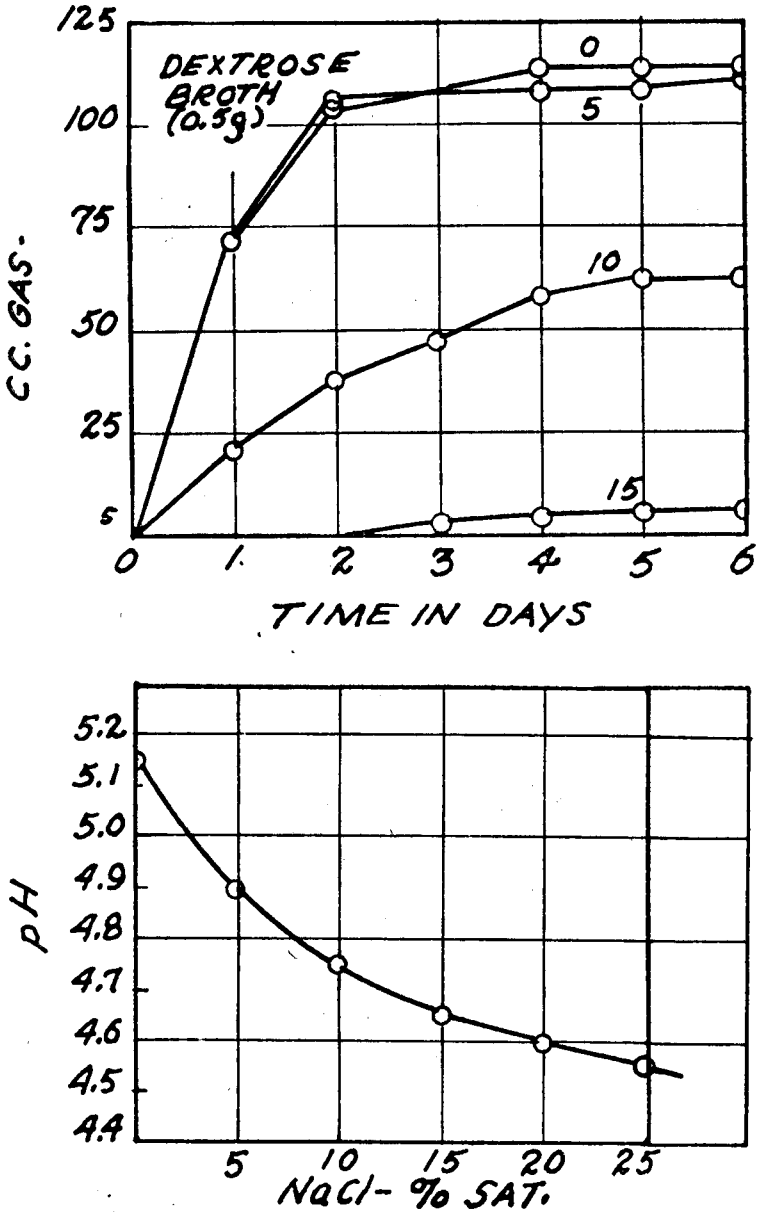

Fig. 8. Upper part: Effect of NaCl upon gas evolution from the fermentation of buffered dextrose broth. Curves identified according to percent saturation of salt. Lower part: Effect of NaCl upon the pH of dextrose broth buffered at pH 5.15.

incubated one week at 35° C. In all fermentations, the residual gas was boiled out and included in the collected gas at the time of the analysis.

The results for the dextrose lots are shown in Fig. 8 (upper part). There was a marked decrease in gas production as the salt concentration was increased above 5-percent saturation. Salt concentrations as high as 20- and 25-percent saturation resulted in no growth. Further investigation indicated that a substantial lowering of the pH of dextrose broth was caused by the addition of salt.

In order to determine the extent of the influence of salt on the pH of this medium a series of dextrose broth samples which were initially buffered at 5.15 was then made up to contain salt equivalent to 5-, 10-, 15-, 20- and 25-percent saturation. The pH values of this series were determined and are shown in Fig. 8 (lower part). This test demonstrated that the incorporation of salt in the medium decidedly lowered the pH value of the medium and that the effect increased as the salt content was increased. From these observations it was concluded that at least a part of the inhibitory effect of salt in dextrose broth upon the growth of strain H-1438 was due to the lowering of the pH value of the buffered medium.

As a further check of this possibility, an experiment was set up similar in all respects to the inoculated dextrose broth series with the exception that cucumber juice broth containing no added buffer was used rather than dextrose broth. The pH of this medium was adjusted to an initial value of 5.9 in order that the addition of salt would not bring the pH into the inhibitory range. The addition of NaCl to cucumber juice medium had very little effect upon the pH of this medium (pH range for salt series—pH 5.7 for 5-percent saturation and pH 5.55 for 25-percent saturation with respect to salt).

In this series the salt had the same general effect upon gas production as previously experienced with the dextrose lots, although adequate growth and gas production resulted in the 20-percent saturation lot. Slight growth was noted in the 25-percent saturation lot but evidently not sufficient for gas production.

The results with respect to gas evolved and the rate of evolution for the cucumber juice lots are shown graphically in Fig. 9 and are calculated on the same basis (50 cc. of 1.0-percent carbohydrate present) to facilitate comparison between the two lots of media used. It is evident that under laboratory conditions, salt above 5-percent saturation exerts a decided effect upon the fermentation which is demonstrated by a reduction in the gas evolved as well as retardation in the rate of evolution. A part of the effect of salt in the dextrose series can be attributed to the influence exerted upon the pH of the medium.

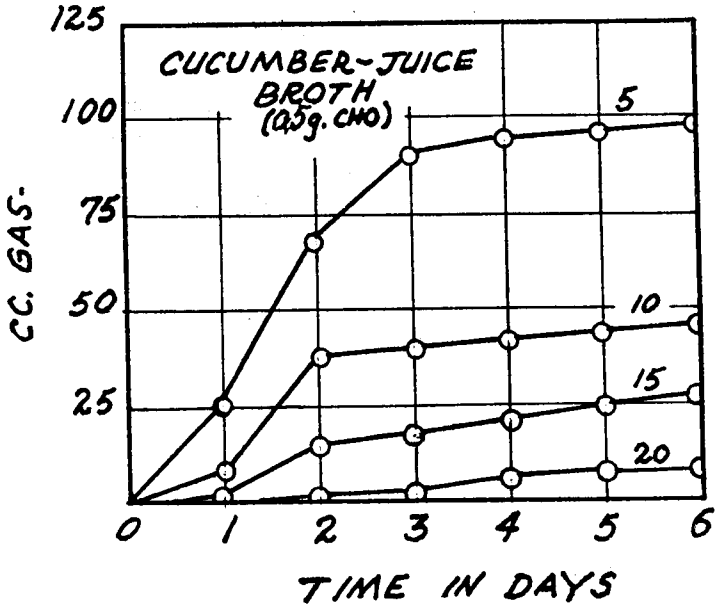

Fig. 9. Effect of NaCl upon gas evolution from the fermentation of cucumber juice broth adjusted to pH 5.9 and without added buffer. Curves identified according to percent saturation of salt.

F. *Fermentation of Various Carbon Compounds*

The preliminary experiments discussed earlier showed that the composition of the gas resulting from fermentations depended upon the carbon source fermented. A comparative study of 14 compounds was made in which the principal aim was to determine the percentage of the components in the evolved gas.

One-percent solutions of the various compounds were prepared and sterilized as previously described. Inoculation and incubation were the same as in previous experiments. At the conclusion of 6 days the collected gas was analyzed. Residual gas was not incorporated at the time of analysis, but was determined after the individual analyses had been made. The pH determinations were made on the test solutions initially (after sterilization) and after the gas analyses had been completed.

Attention is called to the fact that with four compounds, lactose, raffinose, rhamnose and salicin, the 1-percent solutions did not yield sufficient gas volumes for analyses which were comparable with the volumes resulting from the other carbon compounds. Hence, in these cases, the fermentations were repeated, tripling the original amount for each compound so that in the final results the figures are shown calculated on the basis of the fermentation of 50 cc. of a 3.0-percent solution (1.5 g. of compound present).

TABLE 6—*Composition of gas produced from carbon compounds in 0.5-percent tryptone broth by Strain H-1438*

Compound (0.5 g.)	Volume of collected gas	Carbon dioxide		Hydrogen		Ratio of $H_2 : CO_2$†	O_2	Remainder gas	Gas accounted for by analysis	pH of solution	
										At start of fermentation	At finish of fermentation
	cc.	%	cc.*	%	cc.*		cc.	cc.**	%		
l—Arabinose	83.1	59.1	57.4	34.1	28.7	1:2.00	.3	5.4	95.3	6.65	4.6
Dextrose	131.3	66.2	93.3	31.8	42.7	1:2.18	.3	2.2	99.0	6.9	5.05
d—Galactose	111.5	60.8	73.2	36.8	41.5	1:1.76	.5	2.2	99.6	6.9	4.75
Lactose††	46.6	30.6	14.2	64.2	30.4	2.14:1	.3	1.7	97.8	7.05	4.5
Levulose	98.3	63.0	69.0	31.5	32.1	1:2.14	.4	4.9	96.5	6.95	4.55
Maltose	30.5	36.6	15.9	53.3	17.4	1.09:1	.2	2.8	92.9	.....	4.55
d—Mannose	110.8	66.6	79.4	28.1	33.7	1:2.50	.4	5.5	96.7	6.95	4.7
d—Mannitol	131.8	48.6	69.3	50.0	67.1	1:1.03	.3	1.4	99.6	7.15	4.9
Raffinose††	58.7	41.3	24.2	53.7	31.5	1.30:1	.5	2.8	97.2	7.15	4.3
Rhamnose††	52.8	44.6	23.6	49.5	26.1	1.10:1	.3	2.9	96.6	7.0	4.7
Saccharose	109.7	63.8	75.7	29.4	33.5	1:2.25	.7	6.7	96.2	7.05	4.65
Salicin††	58.6	42.7	25.0	52.4	30.7	1.22:1	.2	2.7	97.1	7.05	4.35
d—Sorbitol	85.9	40.0	41.3	56.8	50.7	1.22:1	.4	2.3	99.3	7.1	5.2
l—Xylose	34.6	32.3	18.0	56.0	20.7	1.15:1	.4	3.6	94.9	6.75	4.7

* Figures represent total volumes of CO_2 and H_2 produced from the fermentation and include collected gas as well as residual gas.

** Principally nitrogen from the air present above the cult. medium at start of fermentation, also includes experimental error.

† Ratios calculated from the total volumes of H_2 and CO_2 produced from 50 cc. of 1 percent solutions.

†† Original gas volumes from 50 cc. of 1 percent solution in order listed above were 9.0, 16.8, 9.1 and 13.1 cc. respectively; since these volumes were too small for accurate analyses the fermentations were repeated. Figures in the above table are based on total gas volume from fermentation of 50-cc. quantities of 3 percent solution of each carbohydrate.

The results of the fermentations with respect to gas evolution and composition are presented in Table 6. A relative evaluation* as to the capability of type strain H-1438 to ferment the different substances tested, as evidenced by the amount of gas evolved (excluding residual gas which was similar in amount for all cases where the compounds were utilized to any extent), would be as follows:

Those readily fermented, dextrose, d-mannitol, d-galactose, d-mannose, saccharose and levulose; those less readily fermented, d-sorbitol and l-arabinose; those moderately fermented, maltose and xylose; those poorly fermented, lactose, raffinose, rhamnose and salicin.

In a study of the data concerning the composition of the gas evolved from these fermentations, it was found that the compounds fell into *three general classifications* with reference to production of hydrogen and carbon dioxide. In the first, the gas was composed of 1 volume of hydrogen and 2 volumes of carbon dioxide (1:2). The compounds included in this group were: l-arabinose, dextrose, d-galactose, levulose, d-mannose and saccharose (1 pentose, 4 hexoses and 1 disaccharide).

In the second, the gas was composed of 1 volume of hydrogen and 1 volume of carbon dioxide (1:1). The compounds included in this group were: l-xylose, rhamnose, maltose, raffinose, d-mannitol, d-sorbitol and salicin (2 pentoses, 1 disaccharide, 1 trisaccharide, 2 hexahydric alcohols and 1 glucoside).

In the third, the gas was composed of 2 volumes of hydrogen and 1 volume of carbon dioxide (2:1), and only one compound was included, lactose (disaccharide).

SUMMARY AND CONCLUSIONS

Studies upon the microorganisms responsible for the production of hydrogen in cucumber fermentations have been reported. The experimental work has dealt chiefly with the isolation, identification and biochemical studies of this group of organisms.

Twenty-nine cultures were isolated from cucumber fermentations at brine concentrations ranging from 20 to 60° salometer (percent of saturation with respect to salt). Of the above group of cultures, 20 were isolated during the 1938 season and nine during the 1939 season.

The 20 cultures (1938 season) were studied in detail with respect to morphological, cultural and physiological characteristics. Based upon this investigation, the organisms are placed in the *Aerobacter* genus. Eighteen of the 20 cultures that were given detailed study revealed characteristics in closer conformity to those described in Bergey's Manual for *Aerobacter*

*For the conditions under which this study was conducted and based on the evolved gas from the fermentation of 50 cc. of the various solutions.

cloacae than for those for *Aerobacter aerogenes,* the only other species listed. The remaining two cultures (H-138 and 238) are regarded as varieties of *Aerobacter cloacae.*

A satisfactory apparatus suitable for studying individual gaseous fermentations with respect to gas production, composition and rate of evolution has been described. The advantages of this type of apparatus are (a) simplicity of construction; (b) compactness; (c) ease of handling and manipulation; and (d) its relatively small size, which makes possible the fermentation of a number of samples in a limited amount of incubator space.

The preliminary experiments dealing with the fermentation of dextrose and cucumber juice by strain H-1438 indicated the following: (a) The gas produced from these media was composed solely of hydrogen and carbon dioxide; (b) the composition of the gas from the fermentation depended upon the carbon source (i.e., the ratio of hydrogen to carbon dioxide in the gas from the dextrose fermentation was 1 : 2.3, whereas in the cucumber juice fermentation it was 1 : 5.0); and (c) the fermentations for both media were rapid, the major portion of the gas being evolved during the first 48 to 72 hours.

The dextrose fermentations (buffered at pH 5.15) of seven strains from the stock culture collection showed that with five of the group, the amounts of gas produced, 139 to 148 cc., were comparable. With the exception of one strain, H-138, the gases evolved from fermentations were similar in composition, the hydrogen-to-carbon dioxide ratios being between 1 : 2.32 and 1 : 2.59. However, in the case of the exception (H-138), a difference in fermentation behavior was demonstrated not only by a decrease in the amount of gas produced, but also by a slight increase in the proportion of hydrogen found in the evolved gas (1 : 1.44). Quadruplicate fermentations of dextrose by the type strain (H-1438) showed no significant differences with respect to gas composition which ranged from 1 : 2.35 to 1 : 2.49 for H_2 and CO_2. Also, the majority of the fermentations resulted in practically the same amount of evolved gas, i.e., 143 to 150 cc.

The fermentation of different lots of dextrose at eight maintained temperatures (5°, 13°, 19°, 24°, 30°, 35°, 40° and 45° C.) showed that the optimum was within the 35° range for maximum gas production and rate of evolution. The lower and higher limitations for the fermentation was 5° and 45° respectively. Fermentations maintained either above (40°) or somewhat below (24°) the optimum range of 35° were considerably retarded and less gas was produced. Gas evolution at 19° was much slower than at 40° but at the end of the 8-day incubation period, the amount of gas was about the same. The compositions of the gases evolved from all fermentations were comparable as to percentages of hydrogen and carbon dioxide found.

The fermentation of dextrose took place over a considerable range with respect to initial pH adjustment (pH 5.05 to 9.05) of the medium. How-

ever, the fermentation of different lots of dextrose at several buffered pH values (3.6, 4.25, 5.3, 6.0, 7.0, 7.55, 8.05, and 8.85) revealed that pH 5.3 was the optimum as demonstrated by maximum gas production and rate of evolution. A pH of 3.6 was beyond the limit of growth in the acid range and pH 8.85 seemed to approach the limit of adequate growth in the alkaline range. In general, it was shown by the gas analyses that the percentages of hydrogen and carbon dioxide were similar for all of the above-mentioned fermentations.

Dextrose fermentations (buffered at 5.15) to which salt was added, to the extent of 5-, 10-, 15-, 20-, and 25-percent saturation, demonstrated a progressive decrease in gas production as the salt concentration increased above 5-percent saturation. In this series, salt concentrations as high as 20- and 25-percent saturation resulted in no growth; a part of the inhibitory effect was due to a lowering of the pH below the optimum (5.3) by the action of salt on the buffered broth. Similar fermentations employing unbuffered cucumber juice resulted in adequate growth with measurable gas evolution in the 20-percent saturation lot but not in the 25-percent saturation lot. There were no significant differences noted as to gas composition between fermentations within the same dextrose and cucumber juice series, although there was a considerable difference in the ratio of hydrogen to carbon dioxide found for the gas evolved from the dextrose lots as compared with that from the cucumber juice lots.

A comparative study of the composition of the gases evolved from the fermentation of 14 carbon compounds revealed that the proportions of hydrogen and carbon dioxide depended upon the carbon source fermented. The fermentation of l-arabinose, dextrose, d-galactose, levulose, d-mannose, and saccharose yielded gas composed of approximately 1 volume of hydrogen and 2 volumes of carbon dioxide (1 : 2). The fermentation of l-xylose, rhamnose, maltose, raffinose, d-mannitol, d-sorbitol and salicin yielded gas of approximately equal volumes of hydrogen and carbon dioxide (1 : 1). The fermentation of lactose yielded gas composed of approximately 2 volumes of hydrogen and 1 volume of carbon dioxide (2 : 1). In contrast to the results for the foregoing compounds, the fermentation of cucumber juice yielded gas composed of approximately 1 volume of hydrogen to 5 volumes of carbon dioxide (1 : 5).

PART II.

The *Aerobacter* and Yeast Fermentation of Cucumbers Under Commercial Conditions

By J. L. ETCHELLS, I. D. JONES[1] and F. W. FABIAN

DATA PRESENTED in the previous portion of this bulletin have dealt with the isolation, identification, and biochemical studies of the organisms for the evolution of hydrogen during cucumber fermentations.

The concluding portion of this bulletin presents data obtained in studies conducted at commercial pickling plants on vats of cucumbers salted according to different commercial and experimental treatments. The studies were limited mainly to an estimation of the microbiological activity which occurred in the various fermentations based on the quantitative relationships of the gas evolved. This was accomplished in two ways: by a measurement of the total gas evolved from a representative portion of the vat surface; and by a determination of the composition of the evolved gas based on gas analysis made at frequent intervals.

This report is based largely on studies made during the 1940 season.

EXPERIMENTAL PROCEDURE

In these studies data were obtained from small vats salted according to three experimental treatments under conditions typical of commercial operations and from a number of large vats salted by a commercial company according to their regular schedule.

For the small vats salted according to experimental treatments the general procedure was as follows: Vats of 85-bushel capacity (see Fig. 10) were filled with cucumbers, fitted with false heads, and salt brine was added so as to come to a few inches above the heads. Initial brine concentrations of 20, 40 and 60° salometer (percent saturation with respect to salt) were used. The actual salting schedule, showing the rate of increase of brine concentration for each treatment is shown in Table 7. Throughout this report the salting treatment designation will be the initial salt concentration for that treatment.

[1]Biochemist in Horticulture, North Carolina Agricultural Experiment Station, Raleigh, N. C.

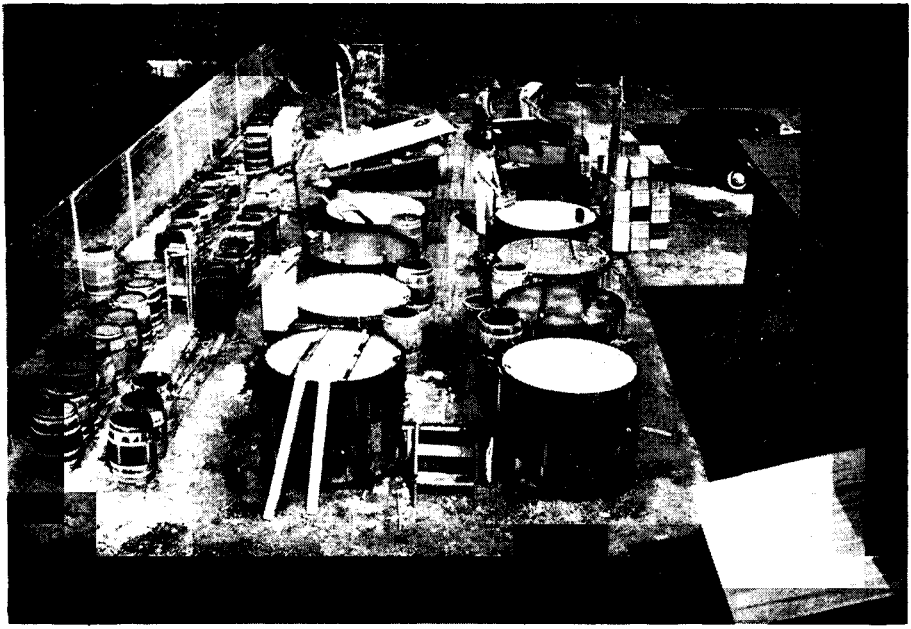

Fig. 10. Experimental vats (85-bushel capacity) of the U. S. Department of Agriculture located at the Chas. F. Cates Company, Faison, N. C. At the time the photograph was taken, most of the vats had been filled and fermentation was under way; two vats (fifth and seventh in right row) are being prepared prior to filling with cucumbers.

In addition to the vats listed above, seven others were studied to the extent of single gas analyses during active fermentation. The latter vats were located at another plant and were salted according to a commercial formula similar to the 40° brine treatment, except that salt and water were added during the filling of the vats with cucumbers in amounts sufficient to make an initial 40° brine.

The prevailing brine temperature during active fermentation (2 to 6 weeks) was within the range of 76 to 80° F. All vats were unsheltered.

The comparative gas evolution studies were based on the amount of gas collected from a representative portion of the surface area of each of the

TABLE 7— *Salting schedule (1940)*

Salting treatment designation and initial brine concentration °salometer	Rate of increase of brine concentration °salometer	Number of vats followed
20	up 10° per week to 60°	2
40	up 5° per week to 60°	3
60	held at 60°	2

small experimental vats. The procedure was as follows: At the time the vats were filled with cucumbers, an inverted stainless steel funnel[2] 14 inches in diameter was placed just below the false head. It was held in place by running the delivery tube end of the funnel through a hole bored in the head. The gases trapped by the funnel were collected over brine in glass bottles of 3- to 5-gallon capacity. At regular intervals the collected gases were removed by displacement into graduated 1-gallon containers and measured to one-tenth of one liter. Gas evolution observations were made from the time the vats were put down until significant gas evolution had ceased (about one month).

Gas composition studies were carried out as described below. For the 40° and 60° salometer treatments the samples were collected from the surface of the brine in the following manner: An inverted glass funnel (4 inches in diameter), supplied with a short piece of rubber tubing and a pinch clamp, was placed over a crack in the false head and the evolved gases were collected by displacement. When approximately 200 cc. of gas were collected, the sample was transferred by displacement into receiving bottles of sampling outfits similar to that shown on the top of the vat in Fig. 11. These gas

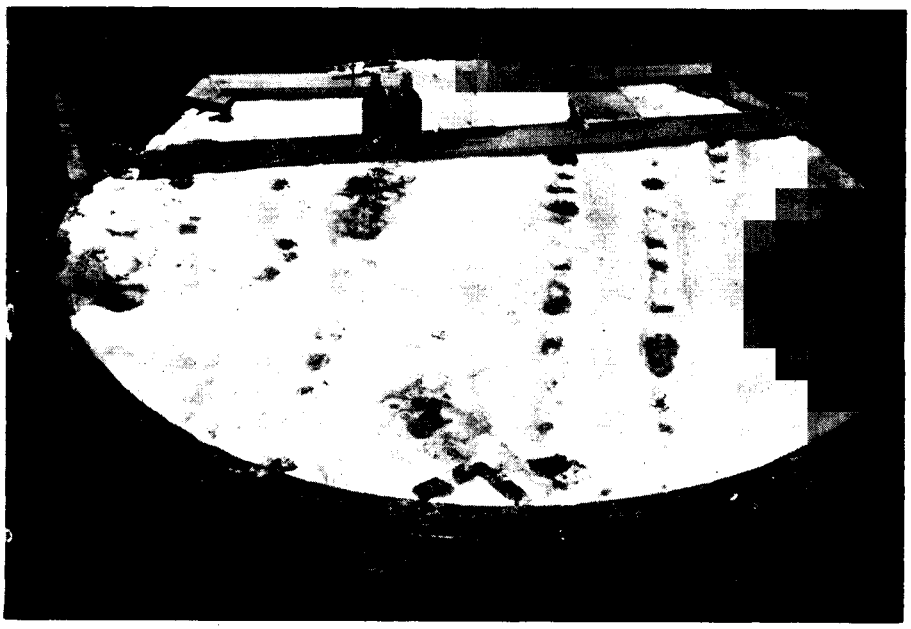

Fig. 11. Typical, vigorous hydrogen fermentation in a 40° salometer brine (Vat 4). Gas sampling transfer outfit (two 12-ounce bottles taped together) is shown at the left on the crossboard on the top of the vat. Just below the bottles, submerged in the brine is the inverted glass funnel used for collecting surface gas sample. The five gallon bottle (full of gas) set over the stainless steel funnel is shown in the background.

[2]The ratio of the portion of the surface area occupied by the funnel (153.9 square inches) to the whole surface area of the vat (4,071.5 square inches) was 1: 26.4.

sample bottles could be readily moved to some location convenient for subsequent gas analysis. The gas analysis for the 20° fermentation was obtained from the 3-gallon capacity collection bottle, used for the measurement of comparative gas evolution. The procedure was essentially the same as that just described.

The method of analysis for the gas samples has been described in detail elsewhere in this report. The determination for oxygen was made on all samples, but the values were small and were considered to be of no significance and are omitted from the results presented. During active evolution, these values always fell between 0.2- and 0.4-percent. During slow evolution of gas from the fermentations, either at the start or during the lag between the hydrogen and yeast fermentations or at the conclusion of the fermentation proper, the values for oxygen increased slightly. This was due to the more or less constant rate of diffusion of air from the cucumber tissue throughout the curing period and, hence, oxygen would show up more clearly when there was slight gas evolution due to microbial activity. Analysis for methane was made on all gas samples and none was found.

Brine samples were taken for bacteriological analysis by inserting a piece of stainless steel tubing through an opening in the head of the vat, down into the brine toward the center of the vat and withdrawing the brine sample through an attached piece of rubber tubing. Two 12-ounce samples were withdrawn before taking the final sample. Samplings in all cases were started from about the time the vats were put down and headed and were continued at regular intervals (1 to 2 days) during the course of fermentation.

All brines were examined at short intervals during the fermentation period in order to determine yeast population. This was accomplished by plating dilutions of the brine on tartaric acid agar (20). This medium consisted of ordinary dextrose agar to which 5 cc. of sterile 5-percent tartaric acid was added to 100-cc. amounts of the agar prior to pouring the plates. Addition of the tartaric acid brought the pH of the medium to approximately 3.7, thus inhibiting all the usual brine organisms except the yeasts. In the case of brine samples from the 60° fermentations, it was found necessary to decrease the amount of tartaric acid to 3 cc. per 100 cc. of medium since the yeasts from the above-mentioned fermentations grew poorly when the greater amount of acid was used. The yeast plates were usually incubated 3 days at 25° C. and counted. In cases where growth was sparse, the incubation period was extended to 5 days.

Two 40° fermentations (Vats 4 and 6) were followed with respect to the populations of the organisms responsible for the evolution of hydrogen (the *Aerobacter*). An estimation of their numbers was based upon the colonial characteristics on nutritive caseinate agar (Difco), containing 8 cc. of 0.4-percent brom-cresol-purple indicator added at the time of preparation of the agar.

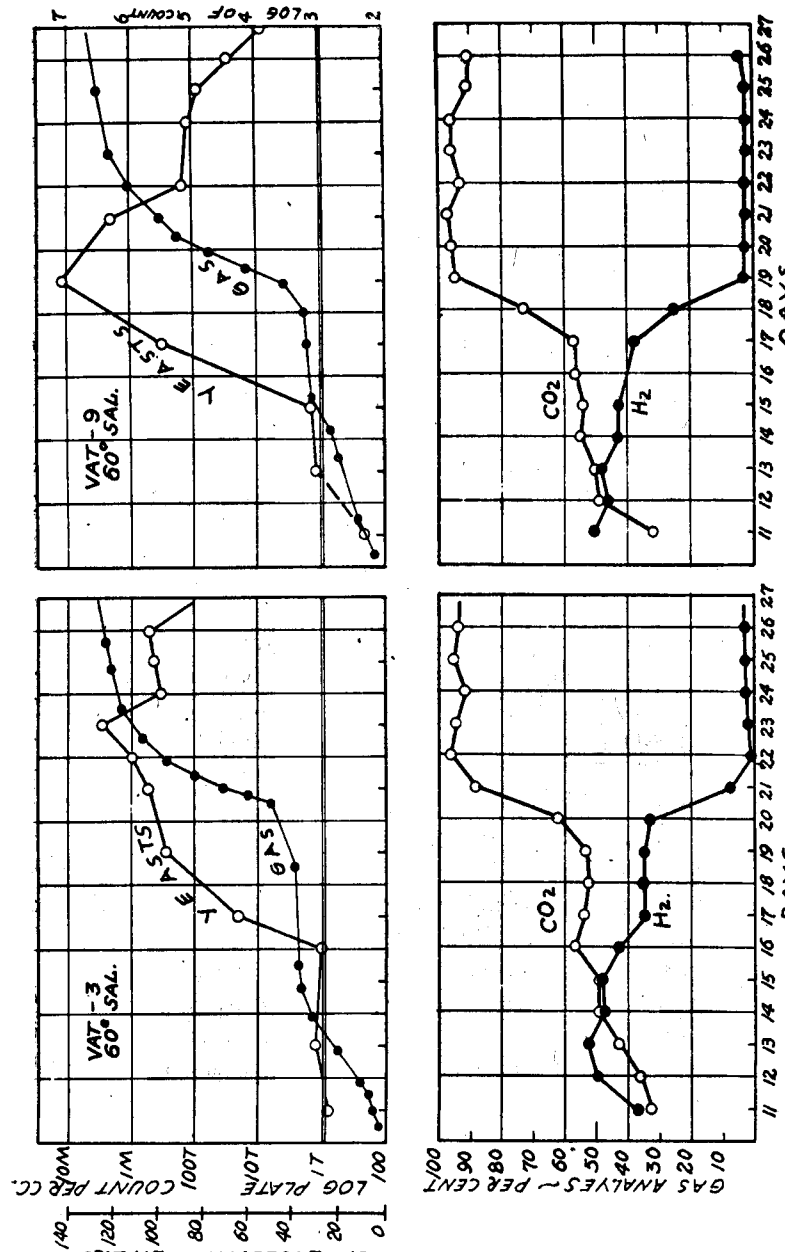

Fig. 12. Yeast populations, gas evolution and analyses of the gas from fermentation in 60° salometer brines (vats 3 and 9).

The results of the studies with respect to volume and composition of the gas evolved and the microbial populations are shown graphically in Figures 12, 13, 14, 16, and 17. In these figures the data are presented in two units, the upper one deals with the populations of the microorganisms and accumulated gas volumes, while the lower one shows the composition of the evolved gas with respect to carbon dioxide and hydrogen.

The values for yeast and *Aerobacter* populations are plotted logarithmically, principally to facilitate showing counts prior and subsequent to active fermentation as well as counts that vary greatly during the active fermentation. Counts less than 1,000 per cc. are shown below the double line drawn parallel to the abcissa, opposite 1T on the ordinate. Values less than 100 per cc. are not plotted.

Values for accumulated gas volumes are plotted on an arithmetic scale in units representing 20 liters. This organization of the data permits a more ready comparison of microbiological activity with population of microorganisms and the composition of the gas evolved during such activity.

GASEOUS FERMENTATION IN 60° SALOMETER BRINES

Over a period of several seasons, probably the 60° treatment shows the most consistent behavior with respect to what might be termed a vigorous hydrogen fermentation. During this phase of the fermentation proper, the evolved gas consists of approximately equal portions of hydrogen and carbon dioxide. The data for duplicate fermentations at the above salt concentration are shown in Fig. 12. The material is presented with respect to progressive changes in yeast population, accumulated volume of gas (upper part), and composition of the gas evolved (lower part). The fermentations in both vats were so strikingly similar that they can be discussed jointly.

An examination of the data indicates that the gaseous fermentation proper, starting on about the 11th day for both vats, was divided into two phases. The first, which covered a period of about 1 week, was brought about by the hydrogen-producing bacteria of the genus *Aerobacter*. Throughout this phase of active gas evolution, it will be noted (lower parts of Fig. 12) that the percentage of hydrogen was relatively high (40 to 50 percent). During the short interval (2 to 3 days) of very slow gas evolution that followed the above-mentioned fermentation, the gas that was collected for analyses came principally by diffusion from the interiors of the "bloated" or hollow cucumbers which were formed during the active hydrogen fermentation. This accounts for the presence of considerable amounts (about 30 percent) of hydrogen during this interval.

The advent of the active yeast fermentation, on about the 16th to 18th day, brought about the second phase of active gas evolution. This is demonstrated by the upward trend of the gas evolution curve. The yeast fermentation covered a period of about 10 to 12 days, which compares similarly with

the period during which a marked increase in the total volume of gas evolved was recorded. During the foregoing fermentation, the gas was composed principally of carbon dioxide although small amounts of hydrogen still persisted owing to continued slow diffusion of gas from the bloaters.

Of the total amount of gas evolved from the fermentations, approximately one-fourth was produced by the organisms of the *Aerobacter* genus, while the major portion was brought about by the yeasts.

GASEOUS FERMENTATION IN 40° SALOMETER BRINES

In general, it has been observed by the authors that the typical, active hydrogen fermentation (see Fig. 11) may or may not occur with the 40° salometer salting treatment. However, during the 1940 season several very active gaseous fermentations at this salt concentration were discovered. This activity occurred during the first few days after the vats were put down. The data from duplicate treatments (vats 4 and 6) are presented graphically in Figs. 13 and 14. In addition to the curves shown for yeast populations,

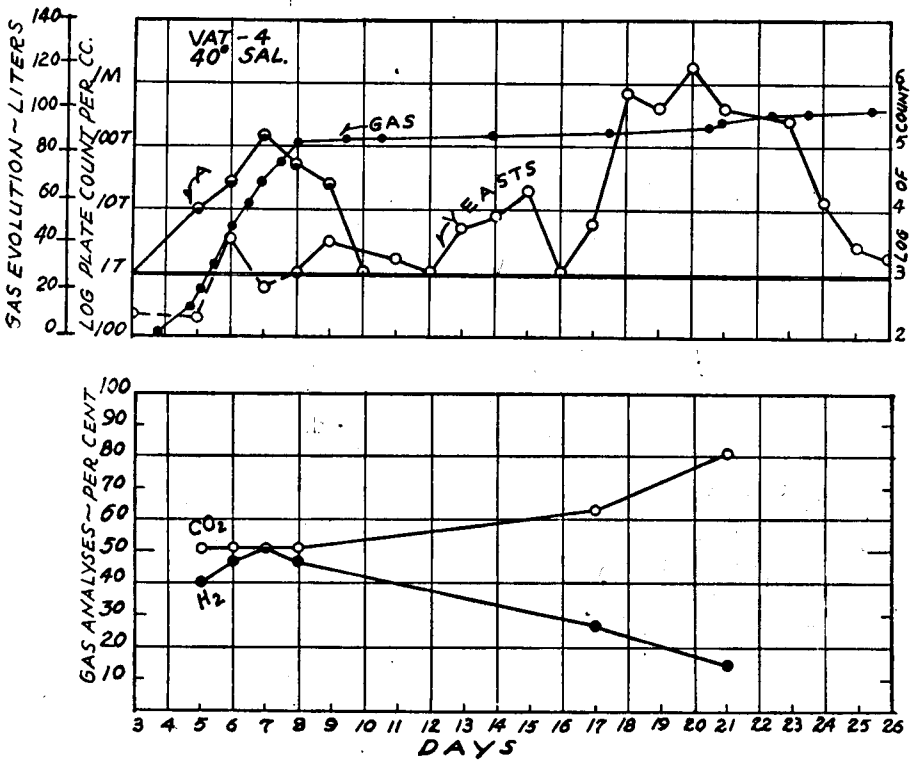

Fig. 13. Yeast populations, Aerobacter populations (curve A), gas evolution and analyses of the gas from a fermentation in 40° salometer brine (vat 4).

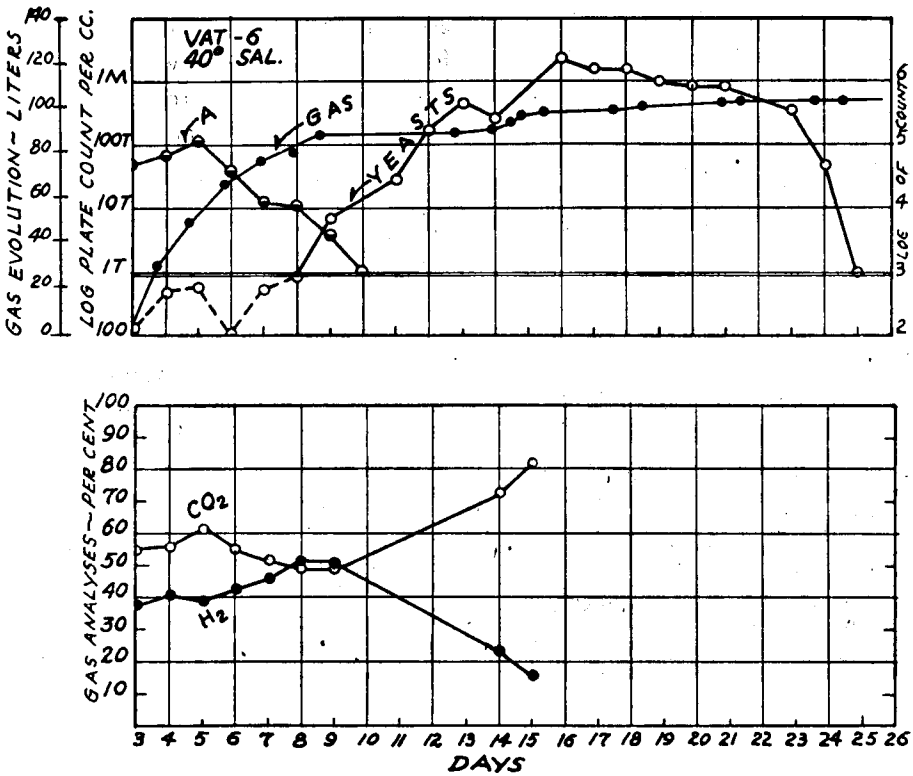

Fig. 14. Yeast populations, Aerobacter populations (curve A), gas evolution and composition from a fermentation in 40° salometer brine (vat 6).

gas evolutions and gas composition,[3] estimates of the number of *Aerobacter* present are also plotted (curves labelled A).

Generalized observations for both fermentations (vats 4 and 6) show that the active hydrogen fermentation started within 3 to 5 days and lasted for a period of about 4 to 6 days. During this period, the gas evolution curves show a sharp upward trend which corresponds with the active growth phase of the *Aerobacter* group in the brine. The composition of the gas throughout this portion of the fermentation proper was similar with respect to proportions of hydrogen and carbon dioxide found present. Near the conclusion of active evolution (8th to 9th day) the proportion reached 1 : 1 (H_2 to CO_2).

It is evident from comparison of the two fermentations in vats 4 and 6 that some variations existed with regard to the onset of the respective yeast fermentations. In the case of the former (vat 4) there was an interval of about 9 days during which gas evolution was very slight, prior to the advent of the yeast fermentation. In the case of the latter fermentation (vat 6)

[3]For these two fermentations, the gas values plotted represent the mean for three or four daily samples taken during the period of the vigorous hydrogen fermentation.

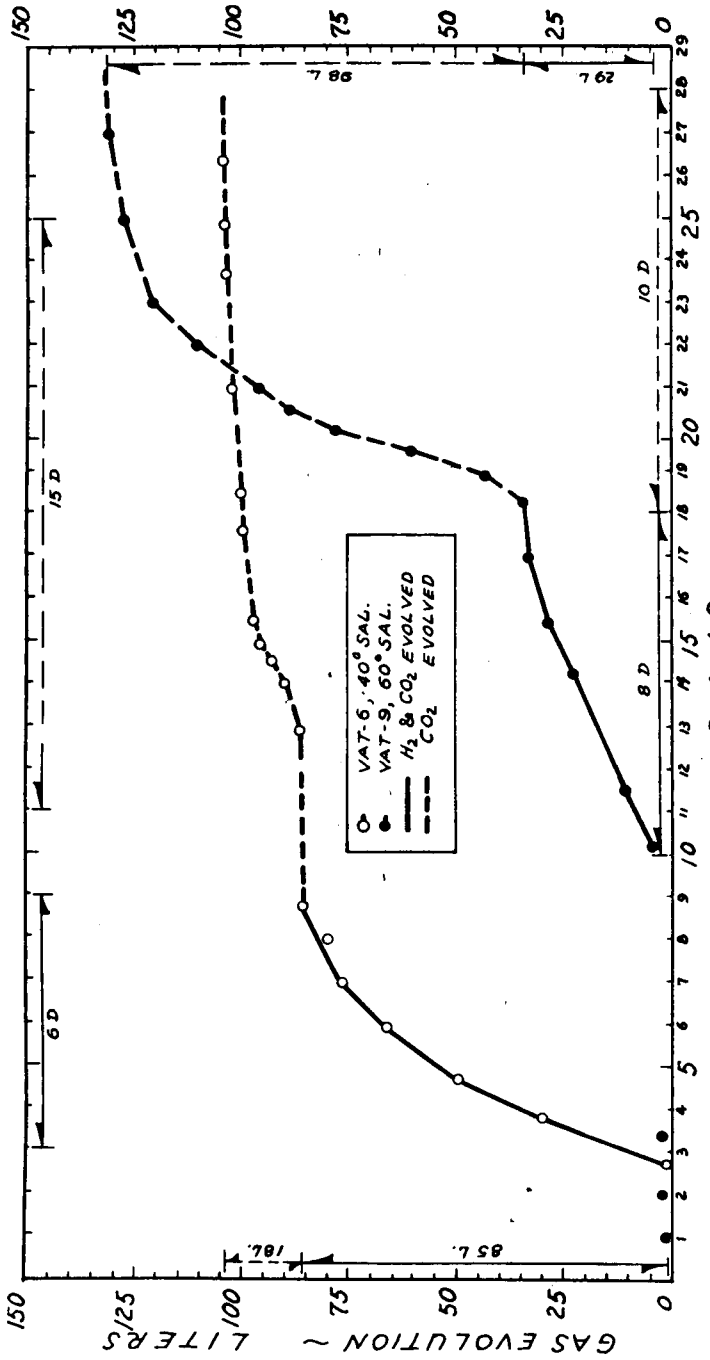

Fig. 15. Comparison of gas evolution from fermentations in 40 and 60° salometer brines. L represents liters of gas collected from the 14-inch diameter funnels; D represents time in days for each phase of evolution showing the H_2 and CO_2 curve for the hydrogen fermentation and the CO_2 curve for the yeast fermentation.

this interval was of about 5 days' duration. However, in both cases, the amount of gas produced by the yeast fermentation represented only a small portion of the total gas produced by the fermentation proper. The analyses of the few surface gas samples that could be collected during the foregoing fermentation showed that carbon dioxide was the principal component. However, small amounts of hydrogen were also found. The presence of the latter was due to the slow diffusion of gas from the interiors of the bloated cucumbers formed during the previous hydrogen fermentation. This was the same relationship previously shown for the 60° brines.

In Fig. 15, gas evolution curves for active gaseous fermentations at 40 and 60° (vats 6, 9, previously discussed) are shown in such a manner as to emphasize the amount of gas produced by each phase of active fermentation as well as the duration of the evolution. This graph shows more clearly what has already been discussed with regard to gas evolution for these two hydrogen fermentations. Based on the amount of gas trapped by the 14-inch funnel, it is noted that for the 40° fermentation, which started on about the third day, approximately 100 liters of gas were collected of which the major portion, about 85 liters, was produced by the hydrogen fermentation (H_2 and CO_2 curve). This amount was produced in about the first 6 days after active gas evolution began. The rest of the collected gas, about 18 liters, came from the subsequent yeast fermentation (CO_2 curve) over a period of about 2 weeks (10th to 25th day). Of the total volume of gas evolved from the 40° fermentations which have been discussed, approximately four-fifths was produced by the organisms of the *Aerobacter* group while the remainder was produced by the yeasts. This is an interesting observation since in the 60° brines almost the reverse was true while in both cases (40° and 60° brines) the yeast counts were similar in numbers and they occurred over comparable periods of time.

In the 60° treatment (Fig. 15) active gaseous fermentation started on about the 10th day. Gas was produced to the extent that approximately 130 liters were collected from the 14-inch funnel. Of this amount, about 29 liters resulted from the hydrogen fermentation (H_2 and CO_2 curve) during an interval of approximately 8 days (10th to 18th day), while the major portion, 98 liters, was produced by the yeast fermentation (CO_2 curve) during the subsequent 10-day period (18th to 28th day).

Thus far, the material presented has dealt entirely with the typical active hydrogen fermentations in 60 and 40° brines. However, it has been mentioned that the latter treatment (40°) may or may not result in the production of considerable amounts of hydrogen. Figure 16 shows the behavior of a 40° fermentation which resulted in only a small amount of hydrogen evolved. Here it is noted that the active yeast fermentation started on about the 11th day and continued until about the 22nd day. During this period there was a gradual increase in the total quantity of gas evolved as shown by the gas

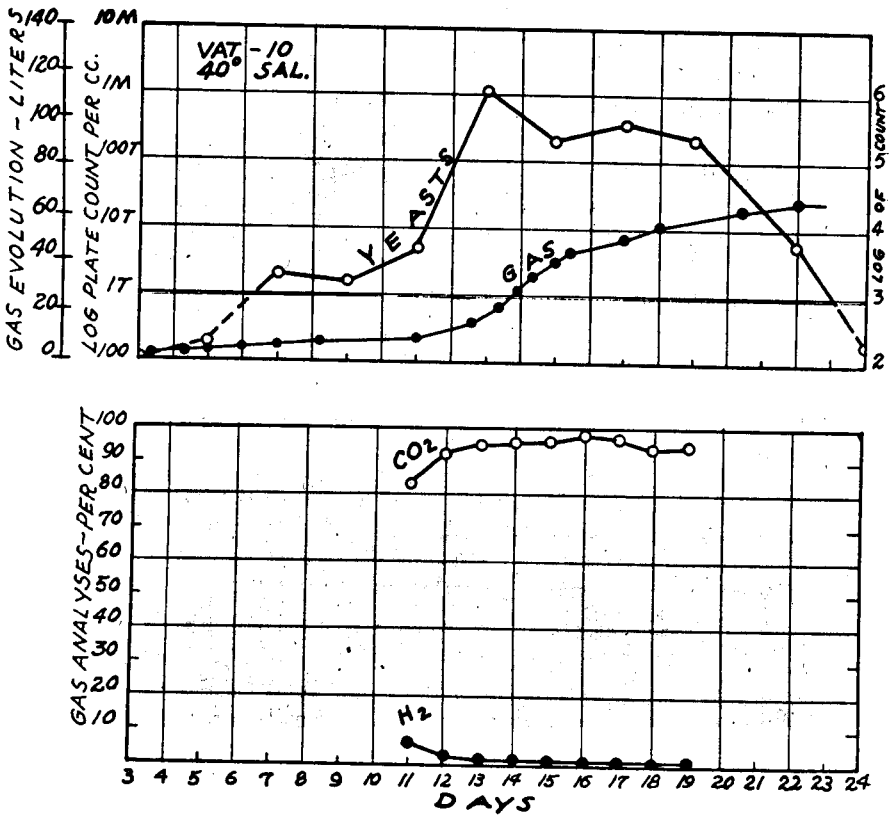

Fig. 16. Yeast populations, gas evolution and analyses of the gas from a fermentation in 40° salometer brine (vat 10).

evolution curve. The gas analyses show that during the active fermentation the evolved gas consisted principally of carbon dioxide, usually well above 90 percent.

The total amount of gas collected from the above-mentioned fermentation was approximately 69 liters as compared with 99 liters from vat 4 and 104 liters from vat 6. The salting treatment was the same for the three vats. The latter two underwent typical hydrogen fermentation whereas the former did not.

GASEOUS FERMENTATION IN 20° SALOMETER BRINES

Some fermentations resulting from the 20° salting treatment may be responsible for small amounts of hydrogen in the evolved gas, while in others this gas may be absent. A typical fermentation at the above-mentioned salt concentration which demonstrates the first behavior cited is shown in Fig. 17. In this case the yeast fermentation started after about the fifth day and continued until the 15th day. The gas evolution curve shows an

upward trend during the active growth phase of the yeasts, although, compared with other fermentations previously discussed, no great amount of gas was evolved. During the early part of the fermentation (third to ninth day), prior to the active gas evolution, it is evident from the gas analysis values that gas other than carbon dioxide and hydrogen was present since the sum of the percentages of these two lacked considerable of being 100 percent. This gas was probably nitrogen from the air bubbles in the cucumber tissue which constituted a considerable portion of the gas sample during the period of slow gas evolution from the vat. However, it is noted that as soon as active evolution started, the percentage of carbon dioxide rose sharply to above 90 percent and stayed in that range until about the 18th day. Small amounts of hydrogen were found during the entire period of analysis. The maximum amount found was 2 percent, while the major portion of the analysis showed less than 1 percent present.

About 32 liters of gas were collected from the above fermentation. This was only about one-half the amount obtained from the 40° fermentation

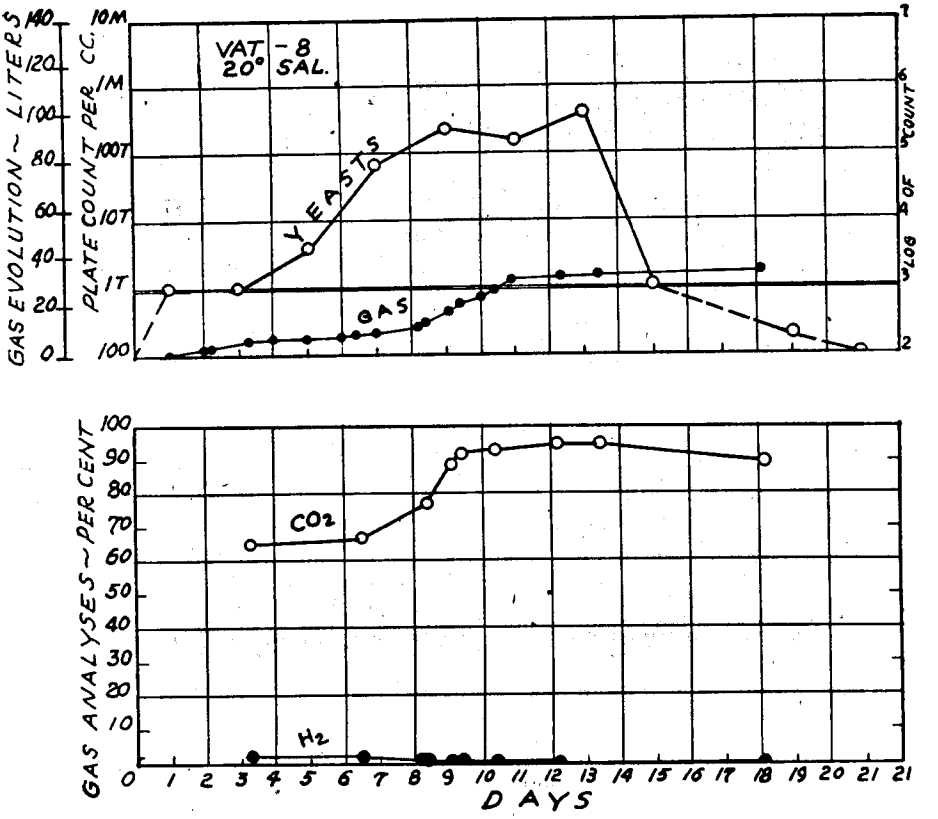

Fig. 17. Yeast populations, gas evolution and analyses of the gas from a fermentation in 20° salometer brine (vat 8).

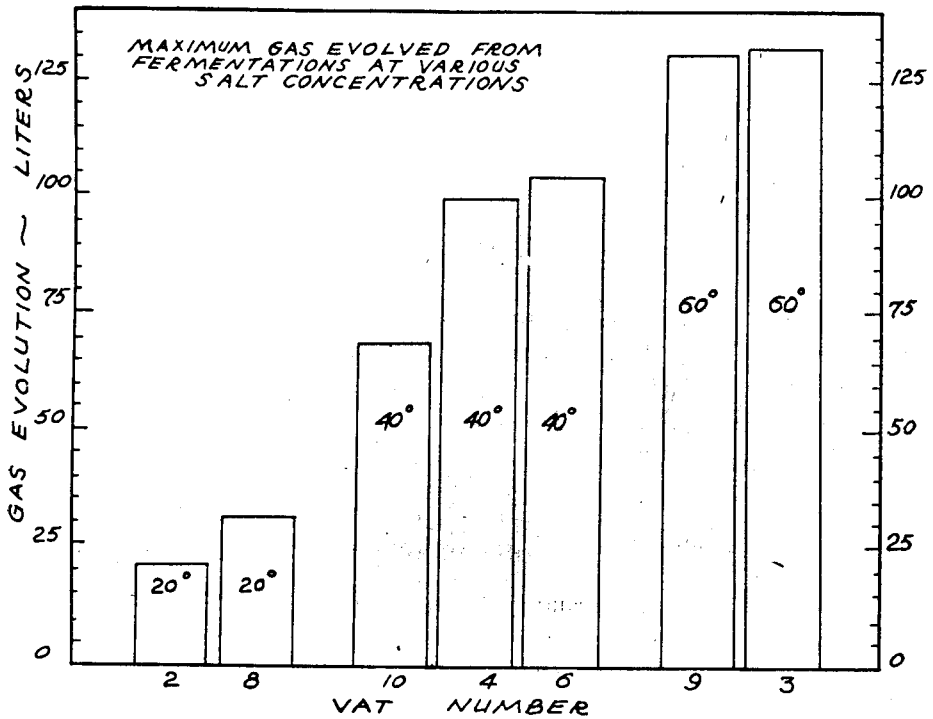

Fig. 18. Comparison of the quantity of gas collected from fermentations in 20, 40 and 60° salometer brines.

(vat 10) which showed similar gas analysis values for carbon dioxide and hydrogen. Furthermore, when compared with the gas collected from the active hydrogen fermentations at 40 and 60°, the 20° fermentations showed only about one-third the amount of gas evolved.

The relationship of the comparative volume of gas produced by fermentation which occur at different salt concentrations is further shown in diagrammatic form in Fig. 18. The gas evolution values shown represent the total gas collected during the period of the first 26 to 30 days after the salting process was started. No further gas evolution occurred subsequent to the period mentioned. In general, it is noted that fermentations at the higher salt concentrations were responsible for the formation of larger quantities of gas. In the 40° series, the two vigorous hydrogen fermentations (vats 4 and 6) resulted in larger quantities of gas than in the case where this phase of the fermentation proper was inactive (vat 10).

Several single analyses from additional 40° fermentations were performed (Table 8). These vats were of approximately 700-bushel capacity and were put down in sequence. All developed vigorous gas evolution within about 3 to 5 days, and the gas analyses showed that considerable quantities of hydrogen

TABLE 8—*Analyses of gas evolved from commercial cucumber fermentations in 40° salometer brines*

Vat	Age	CO_2	H_2
	days	%	%
R1-T2	7	64.2	35.1
R1-T3	6	62.0	35.7
R1-T4	6	56.8	42.0
R2-T1	5	56.8	42.0
R2-T2	7	55.6	43.8
R2-T3	7	62.2	37.8
R2-T4	6	54.4	45.6

were present in the evolved gas from all fermentations. In general, the results shown in Table 8 serve to emphasize the preceding data shown for fermentation at this salt concentration (vats 4 and 6) from which hydrogen was evolved in considerable amounts during the early part of the fermentation.

BLOATERS DUE TO HYDROGEN FERMENTATION

It has been previously mentioned that bloaters or hollow cucumbers were formed during that phase of the fermentation brought about by the organisms belonging to the *Aerobacter* genus. Owing to gas formed within the cucumbers, the latter become distended from gas pressure to such an extent that the three carpels separate and flatten so that often inner cavities of approximately three-quarters the volume of the cucumber are formed. The above-mentioned condition is brought about more often in the large-sized cucumbers although in some instances even the small sizes are likewise affected.

A comparison of the composition of the gas from typical bloaters with that collected from the surface brine during active hydrogen evolution is shown in Table 9. It is evident from these data that the gas showed no significant difference in composition from either source in any of three sets of analyses made.

It was found that the above-mentioned relationship existed only during the short period when actual bloater formation was taking place or at the final point when the bloaters were the most distended due to gas pressure. When the gas from bloaters was analyzed at the conclusion of the active hydrogen evolution period, just prior to the advent of the yeast fermentation, the proportion of hydrogen found was considerably higher. In some instances the increase in the amount of hydrogen was as much as 15 to 20 percent within 2 days following active hydrogen evolution from the brine. This condition was presumably caused by loss of carbon dioxide owing to the greater solubility of this gas in the brine, leaving a greater proportion of the hydrogen inside the cucumbers.

SUMMARY AND CONCLUSIONS

A study of the typical fermentations brought about by the *Aerobacter* under salting conditions typical of the industry revealed several significant observations. Generally, it was found that the 60° salometer salting treatment showed the most consistent behavior with respect to what is termed the typical hydrogen fermentation. Also, it was observed that the 40° treatment may or may not result in the typical, active hydrogen fermentation. Furthermore, some fermentations resulting from the 20° treatment may have small amounts of hydrogen in the evolved gas while in others it may be absent.

The gas evolution, as well as the composition of the gases, demonstrated that typical fermentations in both 40 and 60° brines were divided into two distinct gas evolution phases. The first phase was brought about by the *Aerobacter* group, and during the active period of fermentation the gases were similar in composition with respect to hydrogen and carbon dioxide, the proportion being about 1 : 1. The second phase was brought about by the yeasts, and the gas evolved during the period of their activity consisted principally of carbon dioxide.

In the 40° fermentation, approximately four-fifths of the gas evolved was produced by the organisms of the *Aerobacter* group, while the remainder was contributed by the yeasts. In the 60° fermentation, the situation was reversed; here approximately one-fourth of the gas evolved was brought about by the *Aerobacter* while the major portion (three-fourths) was produced by the yeasts.

A comparison of gas evolution from fermentations in 20, 40 and 60° brines revealed that, in general, the fermentations at the higher salt concentrations resulted in larger quantities of evolved gas.

The gas from "bloaters" or hollow cucumbers, formed during the active phase of the hydrogen fermentation, had practically the same composition with respect to hydrogen and carbon dioxide as did the gas collected from

TABLE 9—*Comparison of the composition of surface gas with that from bloaters or hollow cucumbers*

Vat	Age	Surface gas		Bloater gas	
		CO_2	H_2	CO_2	H_2
	days	%	%	%	%
R1-T2	10	62.2	37.0	62.4	36.1
R1-T3	9	64.4	35.2	58.8	39.8
R1-T4	9	58.5	38.5	57.6	38.2

the surface of the brine. However, this relationship existed only during the actual formation period of the bloaters.

Typical yeast fermentations resulted in all brine treatments (20, 40 and 60° salometer) employed for salting cucumbers. The different salt concentrations did not materially influence the number of yeasts found in the brine. Also, there was no definite correlation between the maximum numbers of yeasts present in the brines and the amounts of gas evolved during their active growth phase.

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