STUDIES ON ACID PRODUCTION BY MEMBERS OF THE GENUS ACETOBACTER

by

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INTRODUCTION

Although the existence of vinegar producing bacteria has been known for many years, classification of this group on the basis of morphological and physiological characteristics was not accomplished until the last decade of the past century. The first extensive studies were made by Hansen (1894), Beijerinck (1898), and Henneberg (1898), who independently classified them into a number of species; the latter were reclassified by Janke (1916). In the most recent edition of Bergey (1939), the classification has again been modified; the vinegar bacteria are placed in a separate family, the Acetobacteriaceae, and the single genus Acetobacter includes fifteen species. Bergey also includes two appendices, one by Henneberg (1898) which lists seven species and the other by Visser'T. Hooft (1925) which admits a new species, namely A. peroxydans (Visser'T. Hooft). Attention should be called to the fact that Bergey et al. (1939), describe all species as possessing in common the formation of a pellicle or film on the surface of suitable liquid media at some period during their development. Vandecaveye (1927), however, isolated and described two active acetic acid producing strains which according to Bergey (1939), would have to be included as varieties of two species already known.

Numerous investigations relative to the influence of various factors upon acidification by acetic acid bacteria have been reported in the literature. Hoyer (1898) and Janke (1916) indicated the course of acidification. The effect of pH on acid production has been extensively

studied by Dratvina (1937), Krehan (1932), and Janke and Kropacsy (1936). The latter co-workers have also shown that the rate of dehydrogenation of alcohol by acetic acid bacteria is directly proportional to the number of cells present. The influence of temperature upon the metabolic processes of the vinegar bacteria has been studied by Waterman (1913) and Wustenfeld (1926). Bertrand and Sazerac (1914), Krehan (1932) and Queré (1933) have shown that salts and their ions may enhance or retard acid production, depending upon the salts employed and their concentrations. The effect of association on acid production when acetic acid bacteria were grown with a variety of yeasts was reported by Vaughn (1936). However, to the author's knowledge, the results reported which concern the effect of aeration upon acid production are very inconclusive. Previous investigations on this latter problem have dealt only with surface aeration and not with the effect of aerating the culture medium itself.

That surface aeration may alter the course of acidification was early recognized. Janke (1916) stated:

> "Particular significance is to be attached to air as an acidification factor. For the oxygen contained therein is necessary for the oxidative process of the bacteria.....if we wish to study the course of acidification then particular care must be given to the consideration of the manner of the air access.....Hoyer has noted that sterile cotton-plugged Erlenmeyer flasks bring about a great irregularity in the gas exchange between the atmosphere, culture flask, and the outer air; the recognition of this same fact led Franzen to replace the cotton plug with a small overlapping vessel. In the course of his acidification experiments with various acetic acid bacteria, Hoyer made the pertinent observation that in Erlenmeyer flasks the acidification proceeds in an irregular manner. According to this observation, particular care must be taken, not only as to the entrance of air into the culture vessel but also to the distribution of the same therein."

Whether aeration can quantitatively affect acid production has received very little study; usually most investigators have given emphasis

only to surface aeration. Vandecaveye (1927) reported that surface aeration retarded acid production perceptibly during the first two months of his experiments but that there was a tendency to the contrary towards the third and fourth months. However, the method he employed was not such as to warrant any definite conclusion. In regard to the results of his experiments Vandecaveye stated:

> "This observation may be incidental, and no definite explanation can be given for it until further work on a more extended scale has been accomplished."

The purposes of this dissertation are to present observations on first, the course of acidification when a suitable medium was inoculated in triplicate with members of the genus Acetobacter; second, a possible definite quantitative affect on acid production when the depths of the cultures employed were aerated, and third, the affect the latter procedure may have on the course of acidification.

ISOLATION AND DESCRIPTION OF ORGANISMS

Cultures were obtained from three sources, namely, fermented cherry cider, muscat grape mash, and mother of vinegar pellicle received through the courtesy of Dr. Fabian of the Michigan Agricultural Experiment Station. Pure strains of the organisms were obtained by repeated streaking on wort agar, Henneberg's agar, and apple cider agar which contained .2% yeast extract, 10% cider and 3% nutrient agar.

Colonies were selected from each of the three named sources. Eight strains were inoculated into 30 cc. of sterile apple cider, which contained 3% by volume of alcohol, to determine the relative acid-producing power of each strain. The strains designated as MC, were isolated from

the muscat grape mash, the CC colonies were isolated from cherry cider, and the FP strains were those obtained from the mother of vinegar pellicle. All cultures were incubated at 37° C. and volatile acid determinations were made upon the fifth day. The results, expressed in grams of acid per 100 cc. follow:

Culture Number	Total Acidity	Volatile Acidity
MC_1	1.31	0.31
MC_2	2.08	1.74
MC_3	2.86	0.90
CC_1	1.66	0.10
CC_2	1.28	0.32
FP_1	1.61	0.01
FP_2	1.66	0.18
FP_3	1.94	0.38

The total acidity of the sterile cider was 0.04.

At the same time that inoculations were made for acid determinations, 10 cc. portions of 0.2% yeast extract in phenol red broth with 3% by volume ethyl alcohol, were also inoculated with one loopful of the organisms to determine the time required for the appearance of acetic acid. The results are tabulated below:

Culture Number	Time to Produce Acid from Alcohol
MC_1	72 hours
MC_2	16 "
MC_3	24 "
CC_1	48 "
CC_2	48 "
FP_1	48 "
FP_2	48 "
FP_3	24 "

The above cultures were inoculated into differential media which contained 0.2% yeast extract and 5% concentration of the substratum to be tested. All produced acid from dextrose and ethyl alcohol but none fermented mannitol, lactose, sucrose, maltose or levulose. The eight

strains were all yellow with iodine. However, there was a distinct difference in pellicle formation. The FP strains all produced a thick tenacious pellicle which could not be destroyed by shaking. The remaining five strains produced a surface film growth similar to that of B. subtilis. For the studies herein reported, the author has selected cultures FP_3 and MC_2, the strongest and most rapid acidifiers. FP_3 is a typical strain of the tenacious pellicle-producing type of Acetobacter and MC_2 is a representative of the film-producing strains.

It may be mentioned here that the upper limits of the isoelectric point of the organisms is approximately in the range 1.2-1.3, as determined by controlled stain technic (Craig and Wilson, 1937). Observations of interest were made when the organisms were stained with borax carmine dye; the cell stained a deep red whereas the supposed carbohydrate capsule was insensible to the reaction. This was confirmed by a modified Molisch test performed microscopically. These observations certainly warrant further work along such lines.

The morphology of the chosen cultures was very irregular and varied markedly with the medium and time of observation. Gram stained preparations from dextrose-yeast extract broth were studied at 16 hours, 24 hours, and daily to the 9th day. In all instances the organisms were Gram variable. They appeared primarily as small rods which occured singly and in short chains, with occasional pleomorphic clubbed and sausage forms. Their morphology on Henneberg's agar displayed long filamentous forms with few encapsulated short rods. It is of interest to note that on dextrose-yeast extract agar, some colonies were found that were entirely Gram negative; however, when transfered to dextrose

broth, at the end of 16 hours these organisms were again Gram variable. Since interest was focused upon acid production, little time was devoted to this problem of morphological variation; our main concern was to secure good acid producing strains representative of the different pellicle-forming types.

EXPERIMENTAL

As previously stated, it is essential that care be taken as to the method of access and distribution of air in the culture vessel to insure an even course of acidification. The author has adopted an arrangement similar to that suggested by Janke (1916). A crystallizing dish of 9 cm. diameter and 5 cm. height was covered by an inverted dish of the same height by 10 cm. diameter. The larger dish was uniformly elevated and laterally separated from the bottom dish by 0.5 cm. The inverted dish was elevated by the support of parallel ledges (0.5 cm.) fixed to a tray arranged to hold the smaller upright dish. Seven of the above described units were employed; three for MC_2, three for FP_3, and one as a control. This technic permitted not only of asepsis but also of an even course of acidification.

The purpose of this part of the experiment was to establish the course of acidification, to determine whether it was paralleled in triplicate identical inoculations, and to use the results for comparison with the cultures to be aerated.

The apparatus employed to study the effect of aeration on acid production is illustrated in Figure 1. The aerated unit was held on a tray similar to that employed for the non-aerated containers. Seven such units were again required.

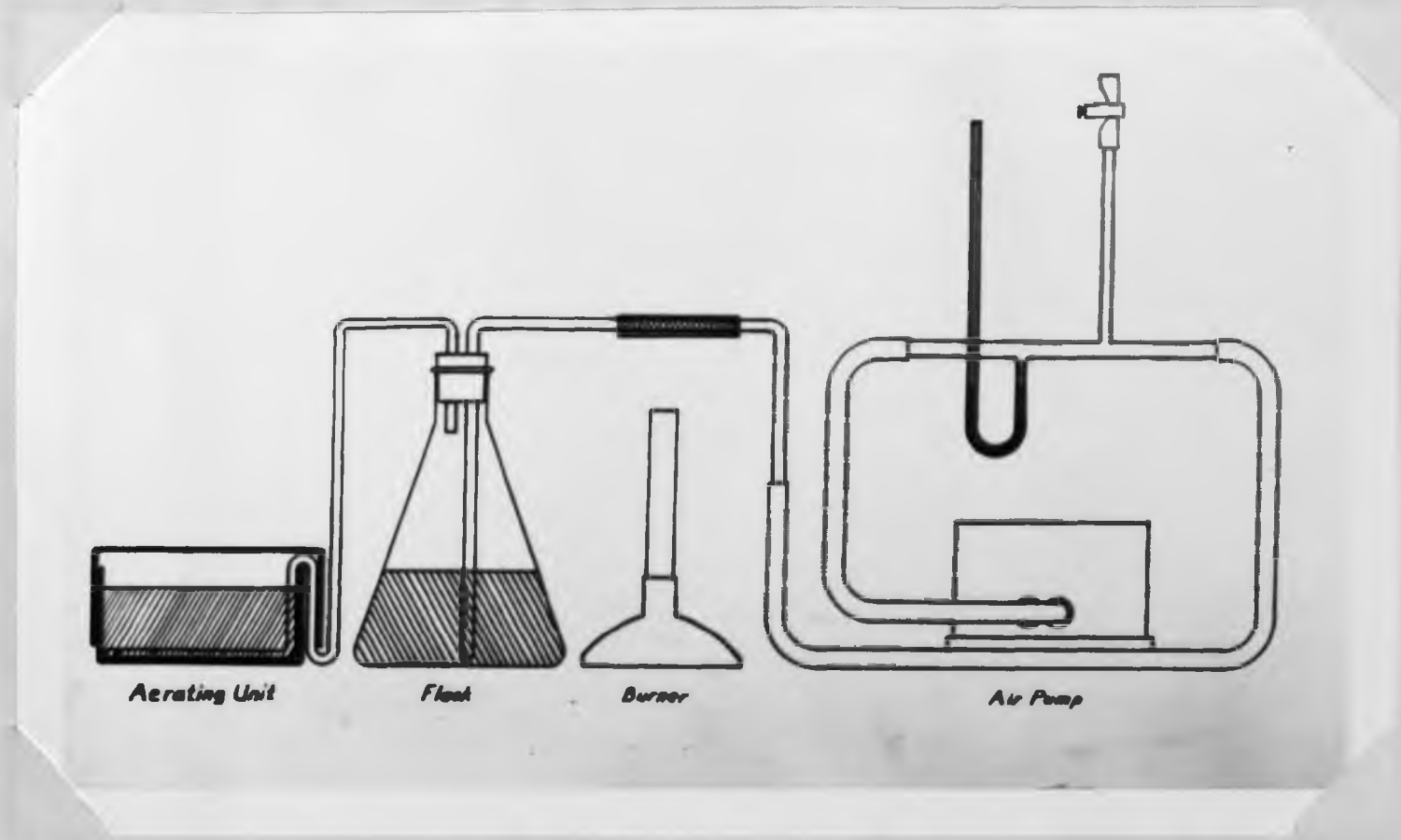


Figure 1. Apparatus employed for aeration. The wooden tray used to separate the two dishes is not included. The volume of air controlled by the screw clamp and capillary manometer passed through the combustion tube heated to red heat. Nutrient broth was contained in the Erlenmeyer flask to cool the incoming hot air and serve as a control for sterility. For the non-aerated part of the experiment the dishes were arranged in an identical manner.

Technic: The units were arranged as described and illustrated and sterilized in the autoclave at 15 pounds pressure for 30 minutes. After sterilization the apparatus was placed in the 37° C. incubator until all of the water of condensation left after sterilization had evaporated. Into each of the 14 crystallizing dishes (9 cm.), 200 cc. of alcoholic apple cider (6% by volume ethyl alcohol added after sterilization) were pipetted, 100 cc. at a time, under aseptic conditions. Hence the height and concentration of media was the same in all of the dishes.

The organisms to be used as an inoculum were obtained from cultures grown in apple cider for 72 hours. The flasks which contained these cultures were vigorously agitated until an even suspension was obtained. In the case of FP_3, the thick-pellicle strain, the more massive parts of the pellicle were removed aseptically with sterile forceps. One cubic centimeter of the MC_2 suspension was inoculated into each of three of the non-aerated dishes and into three of the aerated dishes; similarly one cubic centimeter amounts of the FP_3 suspension were inoculated into three non-aerated and three aerated dishes.

The cultures were incubated throughout the entire experiment at 37° C. The units employed to study the affect of aeration upon acid production, were aerated for 10 minutes each day under the same manometric pressure. For the duration of the 10 minute aeration period the culture dish was slowly rotated by hand, which allowed uniform distribution of air through the medium. Acid determinations were made periodically. A 10 cc. aliquot was removed for each determination, 5 cc. to test for volatile acidity and 5 cc. for total acidity. Titrations were made with N/10 NaOH and phenolphthalein was used as an indicator. The

procedure followed for the volatile and total acid determinations was according to the official method for vinegar.

RESULTS

In a preliminary run of the experiments, all indications pointed to aeration as an inhibiting factor in acid production. Thus the technic was rigidly standardized as outlined previously, and the entire work was carefully performed in triplicate.

The results of the determinations are given below; first in tabular form and then illustrated graphically.

TABLE 1
Culture MC_2 non-aerated

Time in days for determination		2	3	5	6	7	9	11	13	16
Total Acid gms/100 cc.	Expt.1	.40	.44	2.34	3.34	3.85	4.29	4.26	4.33	4.36
	Expt.2	.41	.44	1.89	3.14	3.66	4.48	4.39	4.39	4.37
	Expt.3	.40	.45	1.75	2.43	3.07	4.08	4.21	4.39	4.12
Volatile Acid gms/100 cc.	Expt.1	.08	.14	1.85	3.04	3.45	3.72	3.69	3.73	3.67
	Expt.2	.05	.07	1.57	2.68	3.15	3.90	4.03	3.92	3.67
	Expt.3	.04	.07	1.45	1.94	3.01	3.64	3.78	3.66	3.50

TABLE 2
Culture MC_2 aerated

Time in days for determination		2	3	5	6	7	9	11	13	16
Total Acid gms/100 cc.	Expt.1	.44	.57	1.79	2.65	2.94	3.25	3.33	3.43	3.56
	Expt.2	.41	.56	2.04	2.29	2.62	3.25	3.34	3.42	3.42
	Expt.3	.39	.47	2.21	3.36	3.71	3.85	4.09	4.07	4.14
Volatile Acid gms/100 cc.	Expt.1	.07	.11	1.44	2.15	2.57	2.77	2.81	2.84	2.84
	Expt.2	.06	.07	1.51	1.76	2.22	2.79	2.86	2.84	2.75
	Expt.3	.03	.10	1.97	2.84	3.36	3.44	3.62	3.52	3.72

TABLE 3

Culture MC_2 non-aerated and aerated, averaged for 3 experiments

Time in days for determination		2	3	5	6	7	9	11	13	16
Total* Acid	Non-aerated	.40	.44	1.99	2.94	3.63	4.26	4.29	4.37	4.28
	Aerated	.41	.53	2.01	2.77	3.09	3.45	3.59	3.70	3.71
Volatile* Acid	Non-aerated	.06	.09	1.62	2.56	3.20	3.56	3.83	3.77	3.61
	Aerated	.05	.09	1.64	2.25	2.72	3.00	3.10	3.07	3.10

*Results stated in gms. of acid per 100 cc. of medium.

TABLE 4

Culture FP_3 non-aerated

Time in days for determination		2	3	5	6	7	9	11	13	16	21
Total Acid gms/100 cc.	Expt.1	.41	.47	.50	.48	.56	3.06	4.84	4.82	4.43	3.30
	Expt.2	.42	.41	.47	.47	.55	3.17	4.84	4.67	4.26	2.88
	Expt.3	.41	.43	.53	1.33	2.08	4.68	4.52	4.56	3.80	2.76
Volatile Acid gms/100 cc.	Expt.1	.04	.06	.11	.11	.56	2.76	3.88	3.53	2.66	1.44
	Expt.2	.06	.10	.07	.26	.33	2.50	4.50	3.26	2.60	.97
	Expt.3	.06	.06	.12	.88	1.62	4.04	3.31	2.81	1.98	.66

TABLE 5

Culture FP_3 aerated

Time in days for determination		2	3	5	6	7	9	11	13	16	21
Total Acid gms/100 cc.	Expt.1	.42	.42	.59	1.54	2.50	3.53	3.50	2.98	2.21	—
	Expt.2	.41	.41	.72	1.68	2.82	3.96	3.77	3.58	2.71	—
	Expt.3	.44	.42	.81	1.78	2.61	3.93	3.60	3.28	2.42	—
Volatile Acid gms/100 cc.	Expt.1	.03	.06	.12	1.02	1.97	3.06	2.27	1.09	.23	—
	Expt.2	.06	.03	.33	1.23	2.08	3.27	2.42	1.78	.59	—
	Expt.3	.04	.07	.42	1.36	2.18	3.02	2.42	1.69	.47	—

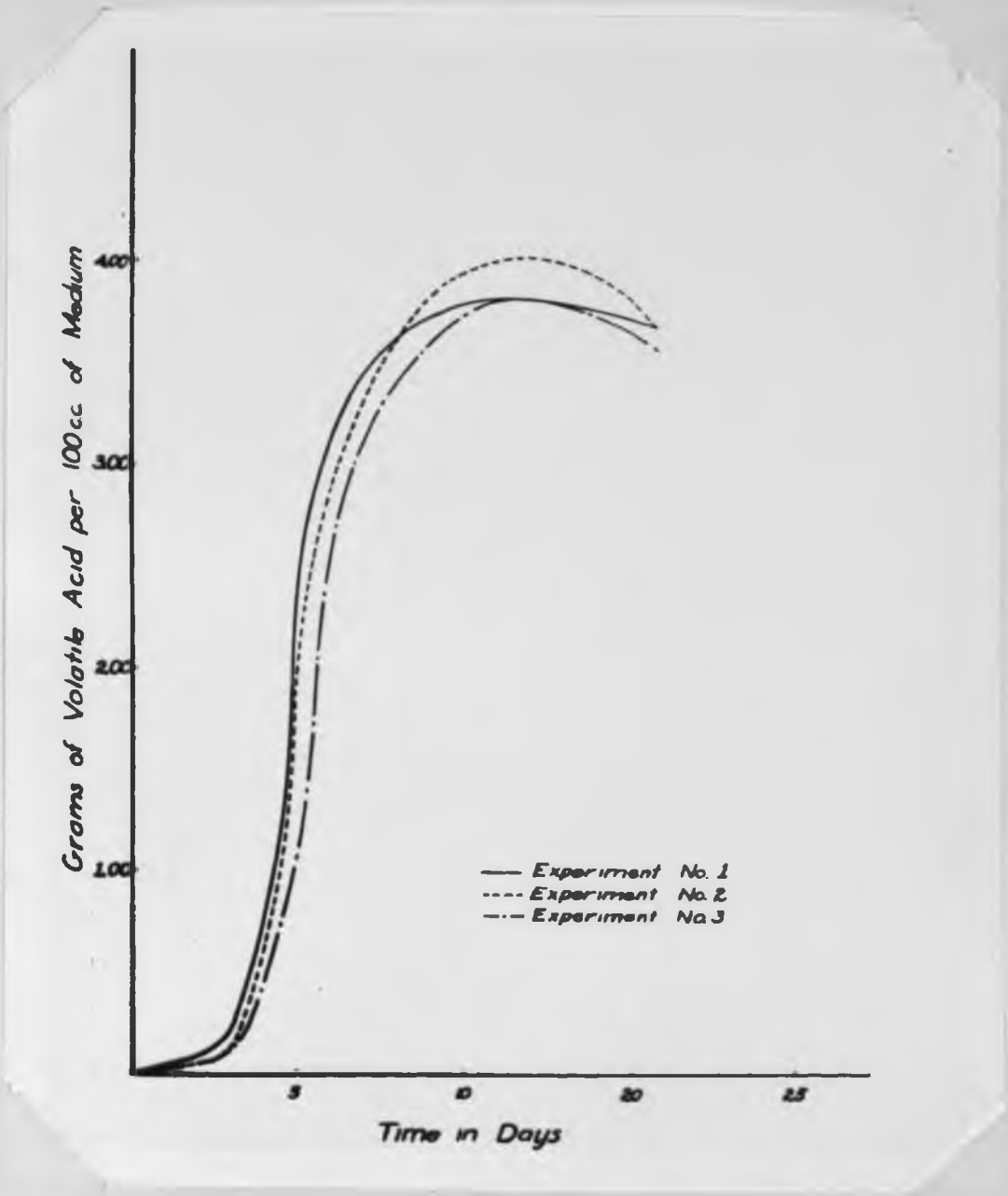
TABLE 6

Culture FP_3 non-aerated and aerated, averaged for 3 experiments

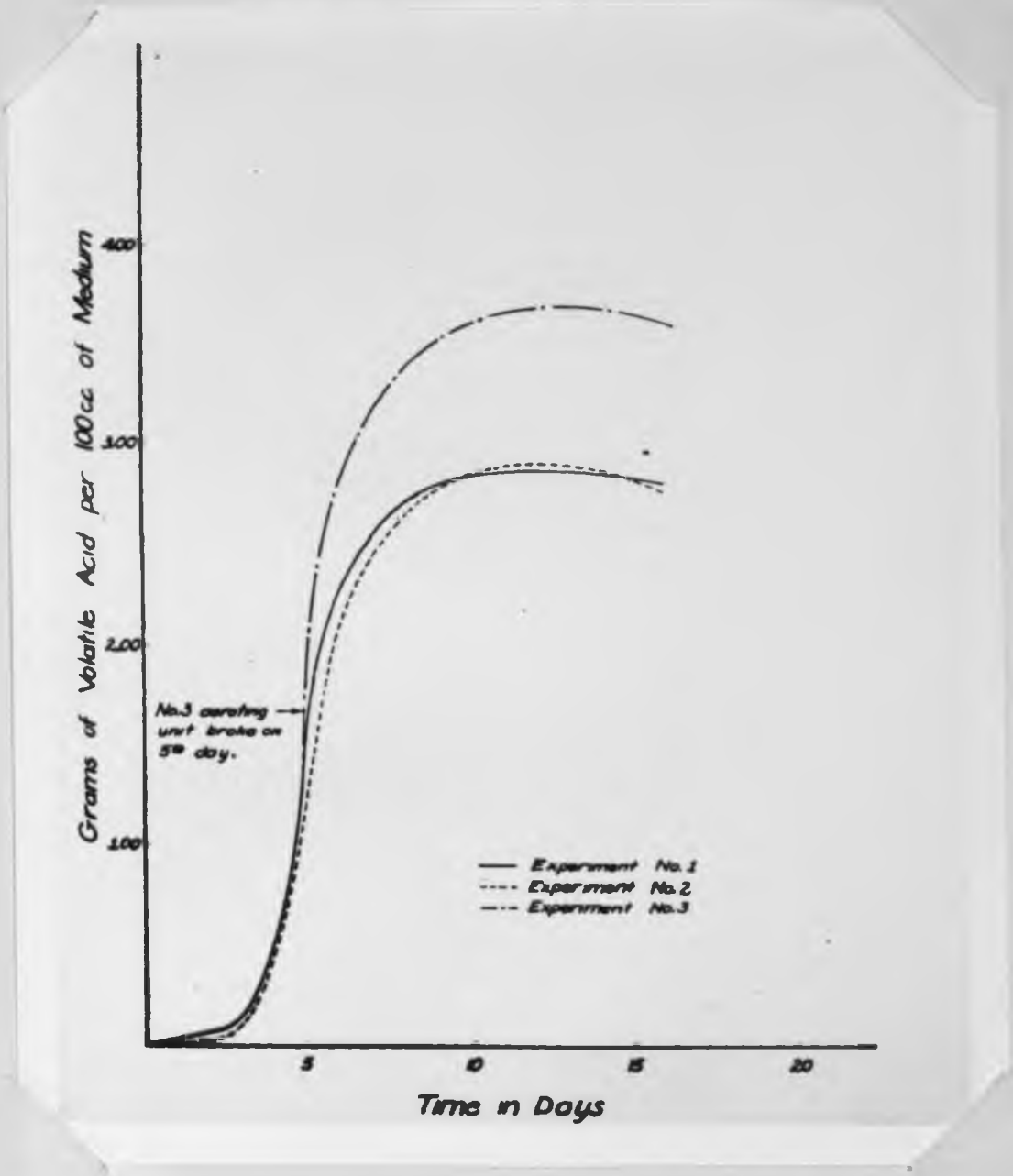
Time in days for determination		2	3	5	6	7	9	11	13	16	21
Total* Acid	Non-aerated	.41	.44	.50	.76	1.06	3.64	4.73	4.68	4.16	2.98
	Aerated	.42	.42	.71	1.67	2.54	3.81	3.62	3.28	2.45	——
Volatile* Acid	Non-aerated	.05	.07	.10	.41	.84	3.10	3.90	3.20	2.41	1.02
	Aerated	.04	.05	.29	1.20	2.08	3.11	2.37	1.52	.43	——

*Results stated in gms. of acid per 100 cc. of medium.

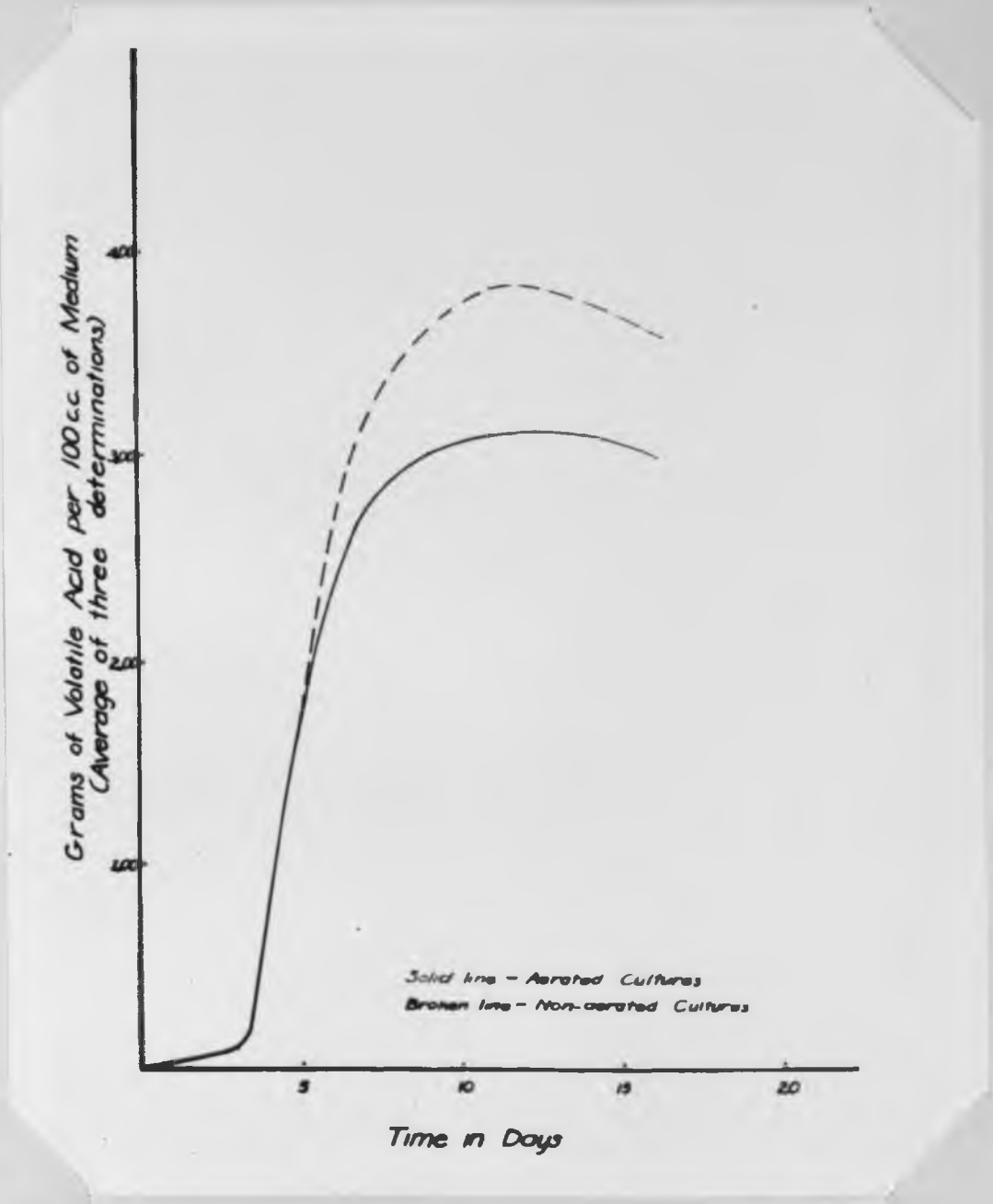
Note: The graphs which follow are plotted from the above tables and are in the same sequence.



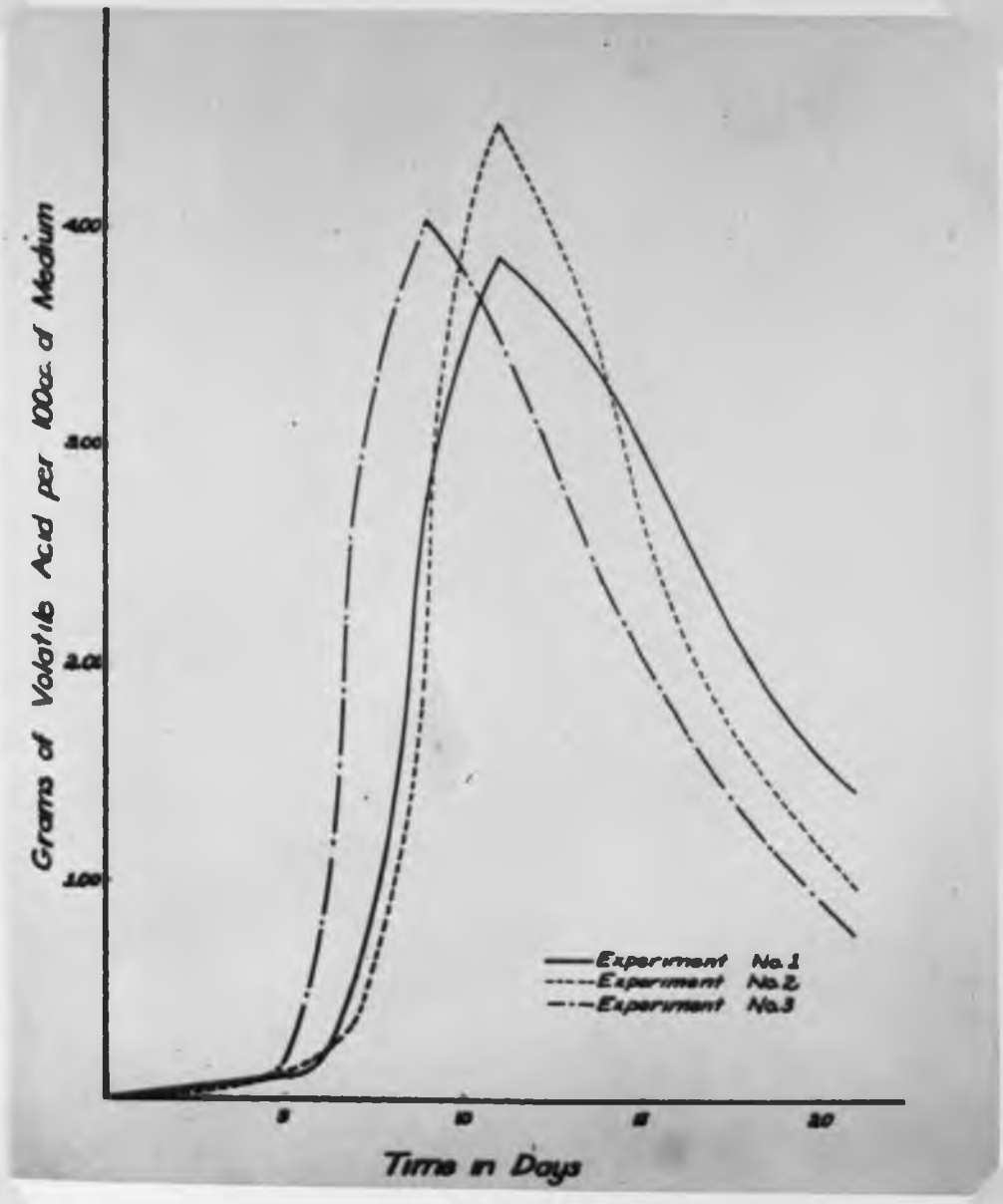
Graph 1. Curves show rate of volatile acid production for cultures MC_2 non-aerated.



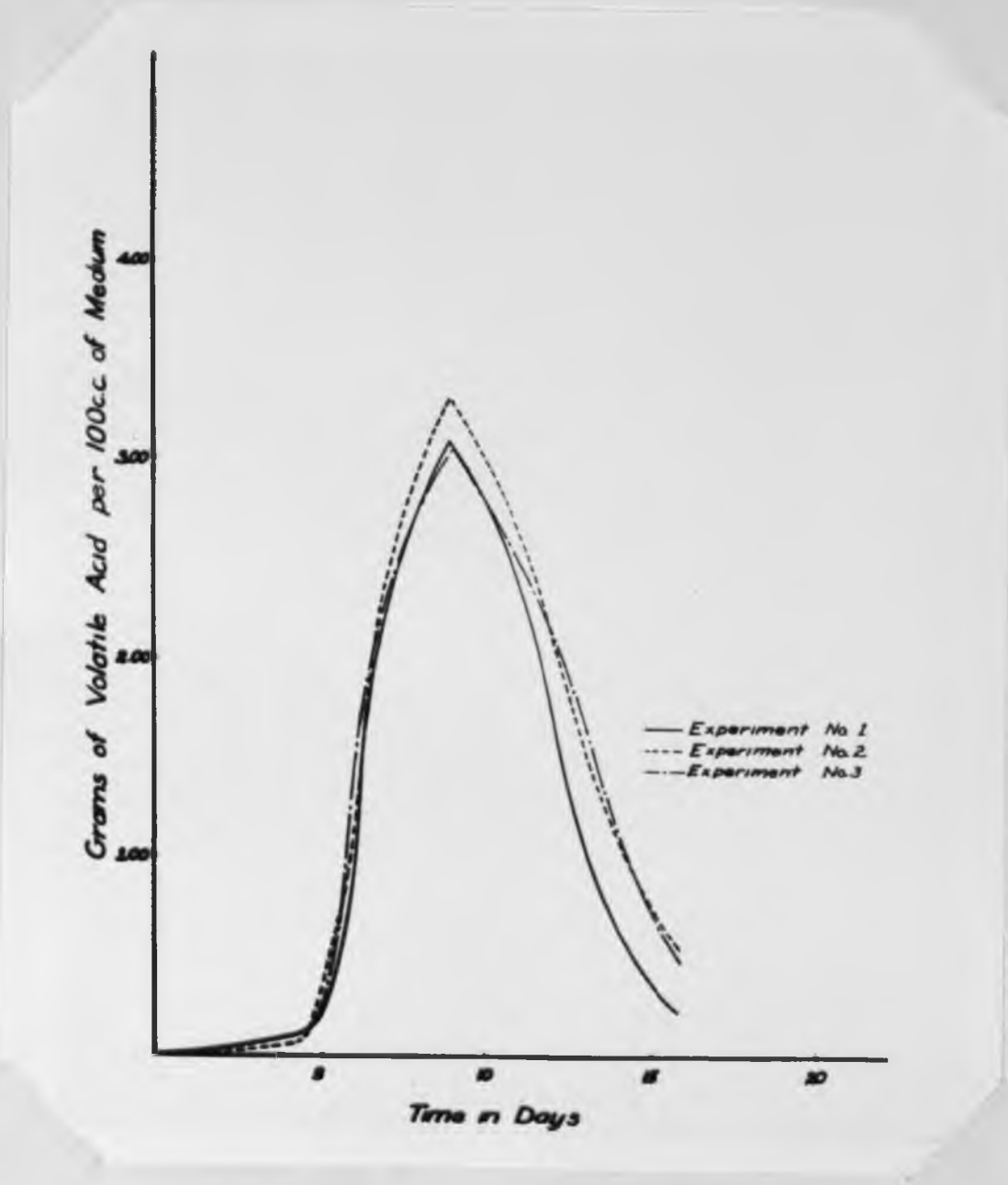
Graph 2. Curves show rate of volatile acid production for cultures MC_2 aerated.



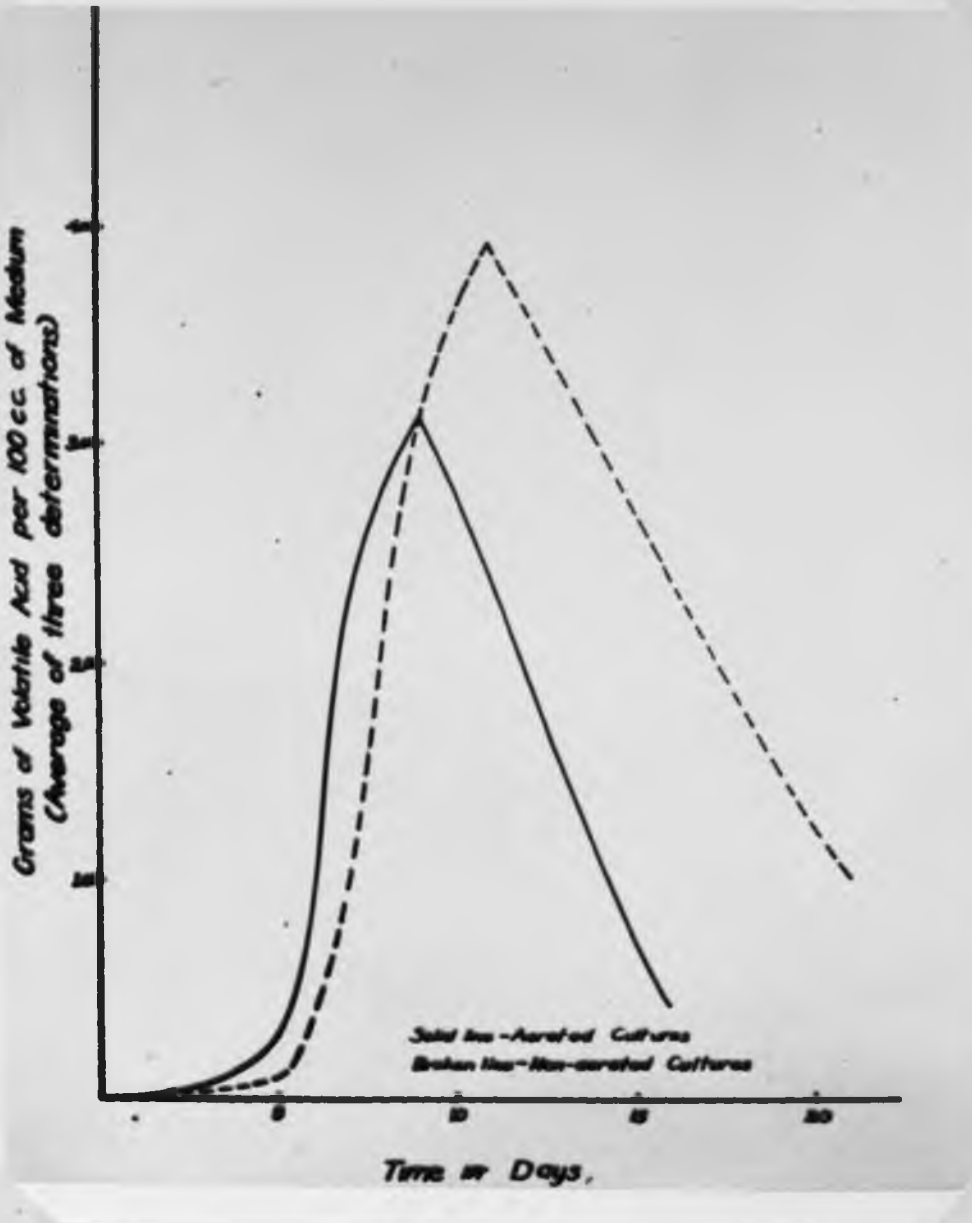
Graph 3. Curves show rate of volatile acid production for cultures MC_2 non-aerated and aerated, averaged for three experiments.



Graph 4. Curves show rate of volatile acid production for cultures PP_3 non-aerated.



Graph 5. Curves show rate of volatile acid production for cultures FP_3 aerated.



Graph 6. Curves show rate of volatile acid production for cultures FP_3 non-aerated and aerated, averaged for three experiments.

DISCUSSION

As was previously established by Janke (1916), the acidification curve consists of three parts; the first being almost horizontal, the second steeply ascending, and the third horizontal or declining. This fact is well illustrated in all of the graphs. Graph 1 clearly demonstrates that the course of acidification is closely paralleled in triplicate identical inoculations for MC_2 non-aerated. In Graph 2, experiments 1 and 2 were very closely paralleled; however, in experiment 3 the aerating unit broke on the fifth day and as the curve indicates acidification was greatly enhanced. This observation strongly supports the fact revealed by Graphs 3 and 6, namely, that aeration of the depths of the culture media has a marked inhibition on acid production. It is seen from these same curves that the maximum yield of volatile acid is decreased by aeration, although there is little effect on the rate of acid production.

It is of interest to note how closely paralleled are the acidification curves, even for the cultures that were aerated, Graph 6. The author believes that this in itself is a good indication of a well controlled technic. In Graph 4 for the FP_3 strain, the three curves would be practically coincidental if the entire curve for experiment 3 was advanced two days and the curve for experiment 2 moved back one day. In this connection, the heavy pellicle characteristic of this organism actually appeared on the surface of the medium, on the 4th, 6th, and 7th days respectively for experiments 1, 2, and 3. There is indication from this, that the speed of acid production is closely correlated with the time of appearance of the pellicle on the surface of the medium.

A comparison of acid production for the two strains shows that their maximum yield of volatile acid is very nearly the same. However, the thick-pellicle organism FP_3 utilizes the available volatile acid very rapidly after it has developed the maximum yield, whereas the film forming organism MC_2 maintains its maximum for several days. With reference to this point, Graphs 3 and 6 demonstrate that MC_2 still maintains its maximum yield of volatile acidity when FP_3 has dropped to approximately 25 per cent. of the maximum volatile acid that it produced.

In all of the experiments with MC_2, aerated and non-aerated, the total acids very closely parallel the volatile acids in the rate and amount of production throughout the entire experiments (Tables 1, 2, and 3). However, for FP_3 the total and volatile acids were parallel to the eleventh day when maximum acidity was attained, after which time the volatile acids dropped off much more rapidly than did the total acids (Tables 4, 5, and 6). This may indicate that part of the volatile acids were utilized by the organisms and part converted into fixed acids.

The observation of most interest in these experiments is in regard to the inhibition of acid production that results from aeration of the depths of the culture medium. This phenomenon was entirely unexpected, as acid production is looked upon as an oxidative process. However, in the case of the MC_2 cultures that were aerated, an accumulation of cells occurred on the sides of the dishes and in the process of dying, turned from their normal white color to a dirty brown. In the author's opinion this dying process may possibly have caused the secretion of inhibitory products which decreased the production of acid. However, this speculation would be invalid in an attempt to explain the findings for FP_3

since in this case the pellicle remained intact. The possibility exists that agitation of the pellicle may in some way interfere with acid production. If this idea is correlated with the assumption, that it is the pellicle surface exposed to the air that is important in acid production, then it is highly suggestive that the latter is decreased when air is bubbled through the medium, as the pellicle slightly submerges and thus would not be in direct contact with the air.

SUMMARY AND CONCLUSIONS

Previous investigations on the effect of aeration on acid production by the acetic acid bacteria, have not led to any definite conclusions. Thus, this work has emphasized the effect of aeration of the depths of the culture medium upon acid production. A technic was perfected which permitted of identical triplicate results in non-aerated as well as aerated cultures.

The apparatus for aeration consisted of an electric air pump which forced air in controlled amounts through a combustion tube into nutrient broth from which it was bubbled through the culture medium.

The work herein reported definitely establishes the fact that with a controlled technic the course of acidification is paralleled when identical containers with the same amount of the same medium were inoculated with equal numbers of certain members of the genus Acetobacter.

In so far as this technic is concerned, aeration had the unexpected effect of decreasing the amount of acid formed, although no appreciable effect was observed on the rate of its production.

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