Observations on a Laboratory Method for Submerged Acetic Fermentation

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Received for publication January 9, 1961

Abstract

Lopez, Anthony (Virginia Polytechnic Institute, Blacksburg, Va.), L. W. Johnson, and C. B. Wood. Observations on a laboratory method for submerged acetic fermentation. Appl. Microbiol. **9**:425–433. 1961.—Submerged acetic fermentation experiments were performed for the purpose of determining the conditions under which this type of fermentation should be conducted under laboratory conditions. The apparatus used consisted of a set of glass tubes provided with air spargers.

Acetobacter acetigenum was found to be the most suitable bacterium among six *Acetobacter* compared under submerged acetic fermentation conditions in a synthetic medium. Statistically significant different rates of fermentation were observed in acetators that were identical in construction, fermentation medium, and aeration characteristics.

Extremely long growth lag periods and complete absence of growth were often observed when starting fermentations. The causes of this behavior were investigated. It was found that it was not produced by lack of nutrients or by presence of a bacteriophage. Different kinds of bacterial starters were studied and compared. Cultures maintained in a liquid medium were reliable starters with a short growth lag period. Liquid medium cultures maintained their good starter characteristics after periods of storage of up to 11 weeks at 40 F (4 C).

A revolution is taking place in the vinegar industry, as it gradually adopts the submerged fermentation process. This fundamental development comes after a period of about 130 years of using basically the same process: the "quick" vinegar generator. In other oxidative fermentation industries, the submerged process has become well established. From the first development in the case of yeast, up to the modern antibiotic production processes, the submerged fermentation procedure has shown superiority to other methods and has made possible an enormous advance in fermentation technology.

Review of Literature

The submerged acetic fermentation process was developed for vinegar production by Hromatka and Ebner. These investigators found (1951*a*) that the method common in the preparation of antibiotics and yeasts could not be used because of the volatility of both ethyl alcohol and acetic acid. Great excess of air had to be avoided; hence, emphasis was placed on uniform distribution of small air bubbles in the substrate. On the other hand, Hromatka and Ebner (1950) found that going below a specific oxygen partial pressure in the substrate causes the respiration of the acetic bacteria to cease. The optimal aeration conditions for obtaining an optimal fermentation rate were found by these investigators. Their findings agree with those of Finn (1954) who states that microorganisms can use only dissolved oxygen in submerged fermentations, and that the rate of supply must at least equal the rate of demand in every portion of the culture liquid.

Hromatka and Ebner (1951*b*) also studied the influence of complete interruption of aeration upon rate of fermentation, and found that 30 sec was a critical time. However, damage to the culture was variable and also depended to a large degree upon acetic acid concentration at the time of interruption.

The optimal temperature was found by Hromatka and Ebner (1953) to be between 24.3 and 29.7 C. The influence of temperature was found to be dependent upon the total concentration of acetic acid and alcohol. Frequent fluctuations in temperature within the optimal range caused a decrease in fermentation rate.

The mashes employed by Hromatka and Ebner were red wines. These investigators (1949) reported that the mashes contained the nutrients essential to the cultures employed. However, except for alcohol content, no data on nutrients required or employed are given.

Experimental Procedures

Description of equipment. Two sets of experimental acetators were built. One set consisted of four glass tubes 70 mm in diameter and 48 in. high. The other set consisted of 12 tubes 35 mm in diameter and 48 in. high. The acetators of both sets were equipped at the bottom with fritted glass filters of fine porosity mounted in rubber stoppers. Compressed air was passed through a pressure reducing valve and an air filter to a surge tank, from which air was fed through the fritted glass filters to each of the fermentation

tubes. Regulation of the air flow to each acetator tube was achieved by needle valves or by screw pinch-type regulators on the rubber tube supply line from the surge tank to the fermentation tube. The size of the air bubbles varied between approximately $\frac{1}{32}$ and $\frac{1}{64}$ in. as determined visually and was controlled by the porosity of the fritted glass filters.

Glass tubing 9 mm in diameter bent in the shape of an "L" was extended upward through the stopper in the lower end of the tubes equipped with a short extension of rubber tubing on the protruding end. Pinch-type clamps were attached to the rubber tubing so that samples could be withdrawn.

Bacteria. All the pure cultures of bacteria used in this work were obtained from the American Type Culture Collection, Washington, D. C. Commercial, mixed cultures were obtained through the courtesy of National Fruit Product Company, Inc., Winchester, Va.

Composition and preparation of bacteriological media. Actively growing stock cultures were maintained on slants of yeast extract agar containing 0.5% Difco dehydrated yeast, 0.3% KH_2PO_4, 2% glucose, and 2% agar. The medium was adjusted to pH 6.0 ± 0.2. Nutrient broth was prepared by dissolving 8 g Difco dehydrated nutrient broth in 1,000 ml of distilled water.

The basal medium employed in the fermentation studies was a modification of the medium used by Litsky and Tepper (1953) composed of the substances listed in Table 1. The pH was adjusted to 7.9 to 7.5. At the start of the fermentations, acetic acid was added to the basal medium to adjust its total acidity to 1.5%.

An enriched basal medium was prepared for later studies which contained the vitamins shown in Table 2 in addition to the components of the basal medium. A separate formulation of the vitamins was prepared and combined into the original body.

TABLE 1. *Composition of basal medium*

Components	Per liter of basal medium
	g
Amino acids (Casamino acids, Difco)	5
	ml
Ethyl alcohol (95% by volume)	130
	mg
p-Aminobenzoic acid	200
Adenine sulfate	20
Guanine hydrochloride	20
Uracil	20
KH_2PO_4	500
K_2HPO_4	500
$MgSO_4 \cdot 7H_2O$	200
NaCl	10
$FeSO_4 \cdot 7H_2O$	10
$MnSO_4 \cdot 3H_2O$	10

Chemical methods. Total acidity was determined by the titrimetric method of the AOAC (1955). It was calculated as percentage of acetic acid per 100 ml of liquid. Alcohol was determined by the distillation-pycnometer method of the AOAC (1955).

Selection of organism. Four fermentation runs were performed for the purpose of determining which one of six *Acetobacter* species was more suitable for submerged fermentation under the conditions that were present. The six species were: *A. acetigenum*, *A. aceti*, *A. xylinum*, *A. ascendans*, *A. pasteurianum*, and *A. suboxydans*. A mixed culture, obtained from a commercial "quick" generator, was also included in the tests.

The fermenting medium for each fermentation run was prepared as follows. A volume of 250 ml of the basal medium was placed in a 35-mm diameter glass propagation tube of identical construction to the fermentation tubes (acetators) described above. Air was fed at a rate to 20 liters per hour. Several slants of actively growing stock cultures of one species were washed into the same tube, and the total acidity of the mixture was adjusted to 1.5% with acetic acid. Total acidity titrations were made every 24 hr. As the acetic acid content increased to 6% as a result of the fermentation, dilutions were made to 2.5%, using basal medium. The temperature in the room in which the fermentation tubes were placed remained at 78 ± 2 F. The temperature of the fermenting liquid varied from 75 to 82 F.

Although the laboratory acetators were not sterilized, they were thoroughly washed and rinsed with distilled water before each fermentation run. Heavily seeded starters were used. The nutrient solutions used were essentially free of bacterial contamination. These factors in addition to the relatively low pH and highly aerobic conditions prevailing during submerged fermentation are highly conducive to the maintenance of an essentially pure culture during fermentation when such an inoculum is used.

Fermentation uniformity trials. Uniformity trials were conducted to determine the base fermentation rate and

TABLE 2. *Additional vitamins used in enriching basal medium*

Vitamin	Concn per liter of basal medium
	mg
Thiamine	1.0
Pyridoxine	2.0
Pyridoxamine	0.6
Pyridoxal	0.6
Calcium pantothenate	1.0
Riboflavin	1.0
Nicotinamide	2.0
Biotin	0.02
Folic acid	0.02
Vitamin B_{12}	0.002

correlation in the rate of fermentation between acetators. The procedure to start the fermentation was similar to that explained under Selection of Organism. When the volume in the small propagation tube had increased to capacity because of repeated dilutions, a transfer of the entire volume was made to a larger propagation tube of 70-mm diameter. The fermentation was allowed to continue in the large propagator until sufficient volume had been obtained to inoculate the battery of 12 acetators. Using this technique, the small fermentors received initial charges of identical inocula.

Each of the 12 fermentors was charged with 250 ml of fermenting liquid from the large propagator. To follow the course of the trial fermentations, a 2-ml sample was withdrawn from each acetator every 24 hr, and the percentage of total acidity was determined. As the percentage of acetic acid increased above 4%, dilutions of the mash with the basal medium were made to lower the acidity to 2.5%, and in later experiments to 3.5%. A second 2-ml sample was taken after dilution and the percentage of total acidity was determined. Data were recorded for the upper level of acetic acid and for the dilution level. Attempts were made to dilute before the acid level rose to 7% in all cases since experience had indicated a lowering of the fermentation rate occurred at high acid, hence low alcohol, levels. Each acetator was allowed to run until a decrease in the rate of fermentation was observed.

A constant air supply of 8 psig was maintained in the reservoir while each individual fermentor was supplied with the maximal amount of air which did not cause excessive foaming. Determinations of the exhaust air flow from the top of the acetators indicated this quantity to be 30 liters or more per hour at all times. Temperature was controlled thermostatically in the entire room in which the experiment was conducted within the range 80 ± 2 F.

Rate of fermentation. Hromatka's work indicates that to obtain maximal rates of fermentation it is necessary to maintain the culture in the logarithmic growth phase. This experiment was set up with the objectives of determining (i) the manner in which fermentation rate is affected by dilution of the culture with basal medium, and (ii) whether, and when, the cultures went through a phase of logarithmic rate of growth. It was thought that the method of dilution used in the work reported here might be changing the oxidation/reduction potential and possibly other factors affecting fermentation rate to such an extent that the rates of fermentation might be significantly slowed down after dilution. This might also establish different fermentation conditions in the several presumably identically treated acetators.

Six working acetators were allowed to reach approximately 5% total acidity, and then each was diluted to 3.5% total acidity using basal medium. Total acidity titrations of the fermenting mash in each acetator were made at intervals of 2 hr for a 48-hr period beginning when the dilution to 3.5% was made.

Effect of concentration of ethyl alcohol upon rate of acid production. Commercial experience indicates that the rate of production of acetic acid is significantly lower when the concentration of alcohol in the fermenting mash is lower than 0.25 to 0.30%. This experiment was performed to determine if low alcohol levels or depletion of the alcohol in the fermenting mash were causing a leveling off in rate of acid production by acetators. During four successive days, 100-ml samples were taken at random from three working acetators. Total acidity and alcohol determinations were made in the samples.

Effect of nutrient content upon rate of fermentation. Another possible explanation of the seemingly erratic production of acid in different identically treated acetators is that the absence, or presence at too low a level, of an essential bacterial nutrient might at times slow down the rate of fermentation. The literature indicates that different species of *Acetobacter* have different nutritive requirements, but with the exception of *A. xylinum* (Litsky and Tepper 1953), little information is found on the specific requirements of the different species.

In different tests, yeast hydrolyzate and a complex mixture of vitamins were added to the basal medium in six acetators, whereas six other acetators were treated identically except that yeast hydrolyzate or additional vitamins were not used. Table 2 shows the additional vitamins and the concentrations. Total acidity titrations were made every 24 hr. Dilutions to 3.5% total acidity were made when the percentage of acetic acid was found to have increased to above 4%. Dilutions of the control acetators were made with basal medium. The other acetators were diluted with the basal medium containing either yeast hydrolyzate or the vitamin mixture.

Determination of presence or absence of bacteriophage. The purpose of this experiment was to determine if the presence of bacteriophage was responsible for the erratic fermentation rates and ultimate slowing down of the fermentation. A suspension was made of cells of *A. acetigenum* by washing actively growing stock cultures off slants with basal medium. A 1-ml inoculum was taken from the suspension and placed in sterile Petri dishes which were then poured with yeast extract agar. The poured plates were incubated at 28 C for 24 hr. Samples of 50 ml were then withdrawn from each of six acetators which were showing rates of fermentation characteristic of those generally found in the course of this work. The samples were filtered through sterile U. F. fritted glass bacteriological filters. A 1-ml aliquot was taken from this filtrate and spread over the surface of the poured plates. The plates were allowed

to incubate for 3 days at 28 C and for 1 week at room temperature. Daily observations were made at the end of the 3-day incubation period at 28 C.

Randomized Gram stains and simple stains were made of the cultures in the tubes, as well as of the growth obtained on the poured plates, to determine whether contaminating organisms were present.

Most probable number and standard plate bacteria counts. A search was undertaken for a fast method by which numbers of live organisms could be correlated with percent acetic acid in submerged acetic fermentations. The "Most Probable Number" procedure given in *Standard Methods for the Examination of Water and Sewage* (APHA, 1955) was investigated. A 10-ml sample was withdrawn from each of six acetators. Subsequent dilutions were made to $1:10^2$, $1:10^4$, $1:10^6$, $1:10^8$, and $1:10^{10}$, using basal medium acidified to 1.5% total acidity. Turbidity was observed both visually and with a photoelectric colorimeter.

Standard plate counts were also made for correlating numbers of bacteria with amount of acetic acid produced. Sterile Petri dishes were poured with yeast extract agar. A 1-ml inoculum of the fermenting mash was placed in the dishes. Triplicate sample dilutions of $1:10^2$, $1:10^4$, $1:10^6$, and $1:10^8$ were incubated at 28 ± 2 C for 2 days after which total counts were made.

Effect of nutrient content and of dilutions of medium upon rate of acid production. Two tests were run, each one with six acetators. In one of these tests the acetators were charged with basal medium, and in the other test with enriched basal medium. The acetators were diluted to 3.5% total acidity when the acidity level had risen above 5%, which occurred within 24 to 36 hr. The fermentation was continued for 11 days in the acetators containing basal medium, and for 10 days in the acetators containing the enriched medium.

Fermentation trials employing the basal medium were conducted to determine what effect repeated dilution was having on the performance of each individual acetator. In these trials the acetators were dumped and recharged daily regardless of the acid level. After an exact 24-hr period a 2-ml sample was withdrawn from each acetator and the percentage of acetic acid was determined. A continuation of these daily fermentations was carried on until the cultures from four propagators had been depleted. Data were recorded for the acid level of the charge and for each individual acetator after the 24-hr fermentation.

Effect of degree of dilution upon rate of acid production. The purpose of this experiment was to determine the influence of different dilutions of fermenting mash upon production of acetic acid. Three sets of two acetators were filled with 300 ml of basal medium. Each set was inoculated with 50 ml of partly fermented vinegar stock, taken less than 18 hr before from a working commercial acetator. The three sets of acetators were identically treated, except with respect to daily dilutions with basal medium. Every 24 hr during the 11 days that the experiment lasted, basal medium was added to the acetators. To two of these, 30% of the volume of fermenting mash was added; to two other acetators, 20% was added; and to the other two, 10%. The dilutions were made in such a manner that the volume of 300 ml was kept constant in all tubes. Total acidity was determined before and after the dilutions.

Comparison of liquid and solid subculturing media as starters. This experiment was designed to find out whether subculturing acetobacter in a liquid medium under submerged fermentation conditions would produce a more reliable starter for the acetator tubes.

This work was thought advisable because acetobacter subcultures used to inoculate the propagation and the inoculation tubes often failed to produce cell multiplication and acetic acid, or failed to produce acetic acid although numbers of bacteria had increased. For this reason many of the experiments which are described above had to be performed several times before results from complete fermentation cycles could be obtained. Therefore, considerably more time had to be spent in each experiment than would be the case if the subcultures would function more reliably under submerged fermentation conditions.

A. acetigenum grown on yeast extract agar slant was transferred into nutrient broth contained in four Erlenmeyer flasks. The flasks were placed in a shaker and shaken continuously for 7 days in a room at 27 C (80 F). A ridge had been blown in the bottom of the flasks to promote more efficient aeration. Total acidity and optical density were determined at intervals during the incubation for the purpose of determining whether there was cell multiplication and acid production. Then the contents of the flasks were combined and placed in a submerged fermentation tube that contained a basal medium of pH 3.5.

The purpose was to observe whether submerged

TABLE 3. *Comparative efficiency of several species of Acetobacter under submerged fermentation conditions* measured as acetic acid produced in the course of fermentation periods*

Species	Run no. 1; 12 days	Run no. 2; 12 days	Run no. 3; 13 days	Run no. 4; 9 days	Avg quantity acid produced per run
	g	g	g	g	g
A. aceti	18.4	11.4	16.6	9.2	13.9
A. acetigenum	23.4	13.2	17.4	11.4	16.3
A. pasteurianum	21.7	12.1	19.0	8.3	15.3
A. xylinum	15.1†				
A. ascendans		9.4	11.4	8.4	9.7
A. suboxydans		8.2	14.1	6.8	9.7

* Temperature of fermenting liquids varied between 76 and 82 F. Air was fed in each tube at a rate of 20 liters per hour.

† Zoogloea produced by *A. xylinum* makes this bacterium unsuitable for submerged fermentation.

fermentation would be started by the shake flask cultures. The experiment outlined above was repeated four times.

Effect of pH of subculturing liquid media upon rate of multiplication of Acetobacter. A mixed culture taken from an operating commercial acetator and subcultured on nutrient agar slants, and a pure culture of *A. acetigenum* also grown on nutrient agar slants were individually transferred into Erlenmeyer flasks containing nutrient broth at pH 3.9, 4.6, 5.1, 5.3, and 6.3, and also into flasks containing basal medium at pH 3.9, 5.0, and 6.2. The pH values were adjusted by means of citrate buffers. For each pH value, four flasks were incubated for 72 hr at 27 C (80 F) under aerated conditions produced by continuous shaking of the medium contained in bottom-ridged Erlenmeyer flasks. Total acidity and optical density determinations were made at the beginning and at the end of the incubation period.

Use of only liquid subcultures as starters. In the course of this work no reasonable degree of success had been achieved when trying to get submerged acetic fermentations started by subculturing bacteria grown on solid media. A liquid culture containing 5% acetic acid was taken from an operating commercial acetator. This commercial culture was placed in two fermentation tubes. The same commercial culture with 2% ethyl alcohol added was placed in two other fermentation tubes. A mixture of five parts of the commercial culture with one part of basal medium was added to two other fermentation tubes. The submerged fermentation tubes were charged within 18 hr after taking the commercial culture. All tubes were titrated daily for a period of 7 days. This experiment was repeated four times.

Reliability of starter cultures. Because of the high degree of success obtained in use of only liquid subcultures as starters, this experiment was designed basically for the purpose of determining (i) the reliability of starter cultures carried in a liquid medium,

TABLE 4. *Uniformity trial fermentations, comparative daily acetic acid production in identically treated acetators*

Day	Acetators in test no. 1						Acetators in test no. 2						Acetators in test no. 3					
	G	H	J	K	L	M	G	H	J	K	L	M	G	H	J	K	L	M
1	0.95	1.00	0.95	1.15	1.05	0.85	2.80	2.95	3.00	3.00	2.60	2.30	1.65	1.85	1.95	1.65	1.65	1.75
2	1.70	1.80	1.85	1.55	1.75	1.65	0.10	0.30	0.20	0.10	0.20	0.30	1.00	1.10	1.00	1.70	1.30	1.50
3	2.00	2.00	2.00	2.00	2.10	1.90	0.60	1.20	0.85	1.10	0.10	0.90	1.60	1.65	1.50	1.60	1.30	1.65
4	0.90	2.15	1.90	2.15	1.95	1.50	1.25	1.85	1.45	1.35	0.80	1.70	1.85	1.95	1.65	1.95	1.70	1.95
5	0.15	0.15	0.05	0.15	0.15	0.05	0.70	0.90	0.95	0.85	0.80	1.00						
6	1.45	1.90	1.40	2.35	1.15	0.40	0.20	0.75	0.65	1.15	0.40	1.05						
7	2.55	3.35	2.85	3.40	3.25	2.80	0.40	1.15	0.95	1.95	0.90	1.40						
8	2.10	0.35	2.66	0.95	3.30	0.70	0.10	0.65	0.90	1.70	0.30	1.20						
9	3.50	3.70	1.75	3.90	2.05	3.60	0.80	2.90	2.70	2.30	1.80	2.40						
10	3.50	3.95	1.80	2.60	2.10	2.55	1.15	0.20	0.45	0.80	0.55	1.00						
11	1.05	0.20	2.50	3.90	3.60	3.60												

TABLE 5. *Acetic acid and alcohol concentrations at several stages of submerged fermentation*

Tube	Day	Per cent acetic acid before dilution	Per cent ethyl alcohol before dilution	Total concn before dilution (*GK*)*	Per cent acetic acid after dilution	Per cent ethyl alcohol after dilution	Total concn (*GK*) after dilution	Unaccounted for (*GK*) between days (*GK* after dilution/*GK* before dilution)
B	1	6.00	0.46	6.46	3.50	4.60	8.10	
	2	5.65	0.47	6.12	3.60	4.57	8.17	1.98
	3	6.30	0.42	6.72	3.70	4.70	8.40	2.05
	4	5.70	1.65	7.35	3.50	5.42	8.92	1.05
	5	7.00	0.35	7.35	3.60	4.28	7.88	1.57
D	1	5.25	1.34	6.59	3.50	4.87	8.37	1.29
	2	4.65	0.53	5.18	3.50	3.99	7.49	3.19
	3	5.20	—†		3.60	4.20	7.80	
	4	5.30	1.23	6.53	3.60	5.86	9.46	1.27
H	1	4.50	2.50	6.00	3.50	5.18	8.68	3.46
	2	4.65	0.85	5.40	3.45	4.15	7.60	3.28
	3	5.25	1.20	6.45	3.50	4.05	7.55	1.15
	4	4.50	0.42	4.92	3.70	4.68	8.38	2.63

* Total concentration (*GK*) is a constant figure obtained by adding the alcohol in volume per cent and acetic acid in grams per 100 ml present at any time during the fermentation.

† Liquid level in acetator too low for sample to be taken.

and (ii) the effect of storage time at 4 C (40 F) upon liquid cultures.

Several gallons of partially acetified mash taken from an operating commercial acetator were placed in storage at 4 C (40 F) in hermetically closed bottles. Eighteen hours elapsed between removal of the mash from the acetator and the time in which the mash was placed in cold storage. A volume of 200 ml of the mash was mixed with 50 ml basal medium and placed in each of three 35-mm diameter submerged fermentation tubes. They were immediately aerated at a rate of 30 liters per hour. The temperature of the fermenting mash was from 78 to 83 F during the fermentation period of 3 to 5 days. Total acidity titrations were made every 24 hr until an increase in total acidity of at least 0.1% was observed in each of 2 subsequent days. If the minimal increase in total acidity was not observed within 5 days, the fermentation run was recorded as negative.

Also within 18 hr after removing the mash from the acetator, Roux bottles containing yeast extract agar medium were streaked with the partially acetified mash. The Roux bottles were incubated at 82 F for 24 to 48 hr. The bacteria from two bottles containing abundant growth were washed with basal medium into 3.5 liters of basal medium and shaken. Several batches of bacteria suspension were thus prepared, and stored at 40 F. Every 7 days 250 ml of suspension were placed in each of three submerged fermentation tubes and treated exactly as was the acetator mash.

A pure culture of *A. acetigenum* was subcultured in yeast extract agar in Roux bottles. The cultures that developed in the Roux bottles were treated exactly as explained above for the cultures obtained from an acetator.

TABLE 6. *Comparison of numbers of organisms with peak acid levels on successive days*

Days	Acetator H		Acetator J		Acetator K	
	Acetic acid	Microorganisms	Acetic acid	Microorganisms	Acetic acid	Microorganisms
	%	× 10⁶/ml	%	× 10⁶/ml	%	× 10⁶/ml
1	3.55	0.0009	4.75	0.0033	4.15	0.0045
2	3.90	0.0032	6.15	0.07	5.70	1.0
3	6.00	27.0	5.15	0.2	6.10	7.0
4	4.15	33.0	6.70	100.0	4.80	5.0
5	3.70	10.0	4.50	7.0	6.30	100.0

RESULTS

The six species of *Acetobacter* used were selected among several dozen well-recognized *Acetobacter* species on the basis of known characteristics and availability.

Table 3 presents the results. *A. acetigenum* produced greater amounts of acetic acid, did not form zoogloea, and remained functional for a longer period of time than did the other organisms tested. *A. acetigenum* was also found to be superior under the conditions of this work to a culture obtained from an industrial "quick" generator.

TABLE 7. *Effect of daily dilution of fermenting media upon rate of fermentation*

Acetator	Mean acetic acid production						General mean, A through F	"F" ratio	
	A	B	C	D	E	F		Acetators	Days
	g	g	g	g	g	g			
11-Day basal medium	4.41	4.48	4.41	4.84	4.68	4.46	4.55	1	8.5246*
10-Day enriched medium	4.37	4.89	4.79	5.02	4.46	4.88	4.74	5.14†	26.86*
Recharged daily basal medium	5.46	5.58	5.46	5.66	5.42	5.65	5.54	1.40‡	Treatment 38.59§

* Highly significant, less than 0.1% probability that the variation between days in this test would happen by chance alone.
† Highly significant, less than 0.1% probability that the variation between acetators in this test would happen by chance alone.
‡ Not significant, greater than 20% probability that the variation between acetators in this test would happen by chance alone.
§ Highly significant, less than 0.1% probability that the variation between treatments (starting at different acid levels) would happen by chance alone.

TABLE 8. *Effect of dilution with basal medium upon acetic acid production during submerged fermentation*

Per cent dilution	Acetic acid produced each day (g/100 ml)											Total acid produced in 11 days
	1	2	3	4	5	6	7	8	9	10	11	
30	2.65	0.70	0.30	0.57	0.30	1.20	1.53	0.35	0.30	0.60	1.30	9.80
30	0.63	0.63	0.69	0.40	0.65	1.70	1.80	0.55	0.35	0.60	0.50	8.50
20	1.95	1.50	0.45	1.80	3.30	1.40	1.83	0.40	0.30	0.60	0.20	13.73
20	2.10	1.15	0.45	1.20	0.80	1.30	1.85	0.55	0.20	0.60	0.10	10.30
10	2.00	2.05	0.50	1.65	1.50	2.20	1.35	0.15	0.20	1.00	0.80	13.40
10	1.55	1.95	0.55	2.90	1.20	2.30	1.40	2.67	0.20	0.50	0.90	16.12

It was thought that trial fermentations, in which each of 12 laboratory acetators received an inoculum identical in all known respects to that received by all the others, would establish a rate of fermentation characteristic of the existing conditions. Several uniformity trial fermentations done above as a part of the fermentation uniformity trials resulted in failure with respect to establishing a statistically significant, uniform rate of fermentation between acetators. The original plan was to study the nutritive requirements for the selected culture by using a very complete nutrient mixture which was known to support the growth of acetobacter, and compare results with those obtained when one nutrient was omitted. A set of 12 identical acetators was to be used for the experiment outlined above, 6 of which would have contained the medium containing all the nutrients, and the other 6, the medium in which one of the nutrients would have been left out. In experimental practice it was seen that such an approach was not possible because of the very large differences in fermentation rates observed in acetators that were identical in construction, fermentation medium, inoculum, and aeration characteristics. Data from three uniformity trial tests are presented in Table 4.

Data obtained above in rate of fermentation did not show growth lag or logarithmic growth phases. Acid was produced at a relatively even rate throughout the 48 hr that the experiment lasted. Results of effect of concentration of ethyl alcohol upon rate of acid production are given in Table 5. These data indicate that alcohol levels were not within the critical low level at any stage of the fermentations.

Another possible explanation of the seemingly erratic production of acid in different identically treated acetators is that the absence, or presence at too low a level, of an essential nutrient might at times slow down the rate of fermentation. A search of the literature indicated that different species of *Acetobacter* have different nutritive requirements, but with only one exception (*A. xylinum*) no information is found on specific requirements of different species. The addition to the basal medium of yeast hydrolyzate and of a complex mixture of vitamins in studies on the effect of nutrient content upon rate of fermentation showed erratic results with respect to rates of production of acetic acid by different presumably identical acetators. No statistically significant increase in rate of fermentation was found when the tubes that contained the additional vitamins and the yeast hydrolyzate were compared with the control tubes.

Results of the experiments for determination of presence or absence of bacteriophage indicated absence of bacteriophage and that acetobacter were not contaminated with other bacteria.

TABLE 9. *Total acidity and optical density values obtained during incubation of Acetobacter acetigenum in nutrient broth under aerated conditions*

Day	Flask no. 1		Flask no. 2		Flask no. 3		Flask no. 4		Acetator	
	% Acid	(a) OD*	% Acid	OD	% Acid	OD	% Acid	OD	% Acid	OD
2	0.18	0.115	0.18	0.059	0.18	0.057	0.18	0.105		
5	0.21	0.155	0.21	0.285	0.21	0.265	0.21	0.385		
7	0.22	0.195	0.21	0.275	0.22	0.400	0.22	0.255		
11									0.07	1.0
13									0.07	1.3

* Optical density determined at 550 mμ.

TABLE 10. *Comparative reliability of three different types of starters used to attempt to initiate submerged acetic fermentation under laboratory conditions*

Run	Culture	Culture storage time (weeks) at 40 F*										
		1	2	3	4	5	6	7	8	9	10	11
1	NFP-59†	+++	+−+	+++	++−	+++	+++	−−+	+++	+−+	+++	+++
2	NFP-59	++−	+++	++−	+++	+++	++−	+−+	+++	−++		
3	NFP-59	++−	+++	+−+	+++	+−−	++−	+++	+++	+++	++−	
4	NFP-59‡ subcultured	−−+	−−−	+−−	−−−	−−+	+−+					
5	NFP-59	+−−	−++	−−−	−+−	−−+	−−−					
6	NFP-59	−−−	−−−	−−+	−−+	−−+	+−+					
7	*Acetobacter acetigneum*§	−−+	−−−	−−−	−−−	−−−	+−−					
8	*A. acetigenum*	−+−	−−+	−−−	−−−	−−−	+−−					
9	*A. acetigenum*	−−−	−−−	−−−	−−−	+−−	−−−					

* Each positive or negative sign indicates success or failure, respectively, in starting submerged fermentation in each of three identically treated tubes.

† Culture taken from an operating acetator. Courtesy of National Fruit Product Company, Inc., Winchester, Virginia.

‡ Same culture as in run no. 1, but subcultured in solid medium and bacteria suspended in basal medium.

§ Culture obtained from American Type Culture Collection, Washington, D. C., subcultured in solid medium and bacteria suspended in basal medium.

Most probable number bacterial counts done at several dilution levels showed no significant differences in turbidity. Results of standard plate counts are given in Table 6. Results show lack of correlation between numbers of bacteria and total acidity.

A statistical analysis done on the data obtained above in effect of nutrient content and of dilutions of medium upon rate of acid production is shown in Table 7. The results indicate that daily dilution to 3.5% total acidity with the basal medium and the enriched medium both affect the rate of fermentation. Within treatments, no variation between tubes was observed when the basal medium was employed. Significant variations between tubes was found when the enriched medium was employed. Results also showed that variations in initial total acidity levels had a significant effect on the rate of fermentation.

Table 8 shows the results obtained in the studies of effect of degree of dilution upon the rate of acid production. A statistical evaluation of the data indicates that there is a highly significant relationship between percentage dilution and mean production of acetic acid during the 11 days the test lasted. The acetators that were diluted 10% produced significantly more acid than the acetators that were diluted 20 or 30%. Those that were diluted 20% produced significantly more acid than those diluted 30%.

Results of experiments on the comparison of liquid and solid subculturing media as starters are presented in Table 9. The data indicate clearly that cell multiplication took place, but that acid production was very little, almost nil.

In studies on the effect of pH of subculturing liquid media upon rate of multiplication of acetobacter, growth was observed only in all basal medium cultures of pH 3.9 and pH 5.0 of the mixed culture bacteria. No growth was observed in any of the other mixed culture treatments, nor in any of the *A. acetigenum* treatments. There was no acid production in any of the mixed culture or *A. acetigenum* treatments.

In the four times the experiment on the use of only liquid subcultures as starters was repeated, active fermentation was observed in all six fermentation tubes.

Table 10 presents the results of the study on reliability of starter cultures. The culture taken from an acetator and used directly to initiate submerged fermentation in the laboratory was considerably more reliable as a starter culture than the other two cultures tested. Some of the species of bacteria present in the acetator mash, may not have grown on the yeast extract agar. This fact may account for part or all of the difference found between the acetator mash starter and the subcultured acetator mash bacteria suspension. The qualities of the acetator mash as a starter for submerged acetic fermentation were not affected by storage time of up to 11 weeks at 40 F.

Discussion

When the research reported here was initiated, the main objectives were to determine some of the nutritional requirements of certain *Acetobacter* species when under submerged fermentation conditions, and to compare rates of submerged fermentation of pure cultures of several *Acetobacter* species. The objectives of the work had to be shifted as work progressed because of erratic fermentation rates obtained under submerged fermentation conditions, and because of difficulties encountered in starting submerged fermentations. Emphasis then was placed on finding a good starter and on factors affecting growth characteristics.

Difficulties in starting submerged fermentations are not only characteristic of laboratory conditions. Industry also finds this problem when fermentation is started in commercial acetators. The problem is of lesser importance in commercial practice because acetators can be kept in continuous fermentation for periods of months. Starting submerged fermentations becomes a major problem when, for research purposes, it is necessary to start them often. The results obtained in the work reported here, indicating that starters for submerged fermentations are more reliable when maintained in a liquid medium, may help obviate the problem. Once the submerged fermentation had started there were no major difficulties found in keeping it going for periods of several weeks. Longer fermentation periods were not attempted.

The most important problem found after fermentations had started was that of obtaining statistically significant equal rates of fermentation in acetators that were identically treated. The solution to this problem may be found in making relatively small dilutions of the fermenting mash and in timing these dilutions with decrease in rate of fermentation. The decrease in rate of fermentation can probably be determined under laboratory conditions by continuous mash temperature readings and also by continuous oxidation/reduction potential determinations.

Several factors influence rate of submerged fermentation in an important manner. The more important of these factors are oxidation/reduction potential, alcohol content of mash, dissolved oxygen content of mash, temperature, type of microorganisms present, and qualitative and quantitative composition of mash from the standpoint of bacterial nutrition. Maximal yields and truly continuous production of vinegar by submerged fermentation might be achieved by maintaining oxidation/reduction potential at the optimal value for maximal rate of fermentation during the whole course of the fermentation. The other factors that

influence rate of submerged fermentation would also have to be adjusted within a range that would make the attainment of maximum rate possible. Research aimed at determining the optimum oxidation/reduction potential for maximal yield of acetic acid under submerged fermentation conditions would be worthwhile. In fermentation practice, it may be possible to maintain the optimal oxidation/reduction potential conditions by continuously charging vinegar stock in the acetator, and continuously discharging finished vinegar. It might be possible to do this by using solenoid charge and discharge valves and variable stroke proportioning pumps actuated by differences in oxidation/reduction potential.

There is a definite need for more basic information on the physiology of the Acetobacter, particularly as it concerns the factors affecting the starting of acetic fermentations under submerged conditions. There is scant information available on nutritive requirements of acetobacter. This lack of information becomes more critical in relation to the commercial production of white vinegar than to cider or wine vinegar. Commercially, mixed cultures of acetobacter are used both in the generator and in the submerged fermentation processes. Investigation of possible advantages in the use of pure cultures for submerged fermentation processes has merits, as the equipment is well suited to keeping cultures pure.

Information of the type mentioned above, both in regard to a continuous process and to physiology of the acetobacter, would probably contribute to higher yields and certainly to a better understanding of the process.

ACKNOWLEDGMENTS

The authors are grateful to C. Y. Kramer and D. C. Hurst, Department of Statistics, Virginia Polytechnic Institute, for the statistical analysis of some of the data; to Max F. Bocklet for assistance in constructing one of the acetator sets; and to National Fruit Product Company, Inc., of Winchester, Va., for supplying commercial cultures of acetobacter.

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