A STUDY OF SOME FACTORS AFFECTING GROWTH AND OXIDATIONS OF ACETOBACTER ACETI

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A STUDY OF SOME FACTORS AFFECTING GROWTH AND OXIDATIONS OF *ACETOBACTER ACETI*

By

Henry Paul Meloche, Jr.

AN ABSTRACT

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ABSTRACT

A study was made of factors affecting the growth and oxidations of Acetobacter aceti in mineral medium containing ethanol. It was found that the organism would not grow unless dextrose was included in the mineral medium. In a mineral salts dextrose medium, growth was stimulated by the presence of pantothenic acid, folic acid, thiamine and biotin. However, the acid production of only one of the two strains studied was stimulated in the presence of the above vitamins. There was no stimulation of the organism by amino acids.

Cell suspensions of both strains of A. aceti oxidized ethanol to acetate, but would not oxidize dextrose, gluconate, pyruvate, or acetate. The addition of the vitamins listed above did not "spark" the oxidation of either dextrose or acetate. However, evidence was presented which indicated that cell suspensions grown in a medium containing dextrose and yeast extract would completely oxidize ethanol and acetate, and partially oxidize dextrose.

Experimental pilot plant vinegar generators were inoculated with A. aceti, started and maintained on a mineral medium containing dextrose and ethanol. This study indicated that the major factors to be controlled in the generators were the concentrations of ethanol and dextrose, and the air supply. Vinegar eels did not affect the rate of vinegar production in normally functioning generators.

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A STUDY OF SOME FACTORS AFFECTING GROWTH AND OXIDATIONS OF *ACETOBACTER ACETI*

INTRODUCTION

The acetic acid fermentation is one of the more commonly known biological phenomena. It serves an important role in nature's conservation of carbon, and from earliest recorded history vinegar has been important to man as a condiment. In recent years, vinegar has become a very important economical item to the food industry. Also, this fermentation is often responsible for spoilage of alcoholic foodstuffs.

Most of the vinegar produced in the United States is manufactured by the generator process, also known as the quick or German process. The equipment used consists essentially of a cylindrical tower filled with a material such as corn cobs, coke, beechwood shavings, *etc.*. This material presents a large surface area on which the bacteria grow and provides for maximum oxidative conditions. It is desired that the supporting material impart no flavor or color to the vinegar. In addition, the tower has provisions for the distribution of the substrate to be fermented, the collection of vinegar, and aeration of the system. Vinegar can be made in generators either by the "one run" or tandem processes. In the former the alcoholic stock is completely converted in one generator, and in the latter the stock is passed through a number of generators until the desired acidity is reached.

Most producers making grain or distilled vinegar incorporate a "bacteria food" with their alcoholic substrate in order to supply

nutritional materials which their medium lacks. However, the nutritional value of these "bacteria foods" in relation to the acetic acid bacteria is undetermined, and thus their importance is questioned. In addition, they are expensive.

It is known that some species of the acetic acid bacteria, Acetobacter aceti in particular, can thrive in mineral media. Therefore, it would appear to be an ideal organism to use in the production of grain or distilled vinegar. However, A. aceti along with Acetobacter xylinum is considered undesirable in a generator because both reportedly will completely oxidize any carbon source which is available to them. However, if this overoxidation could be controlled, it might be possible to start and maintain a generator with a mineral-alcohol medium inoculated with A. aceti. This would amount to a considerable economic saving by eliminating unnecessary additives. Finally, generators operated on a mineral medium would provide basic information on the mechanism of the acetic fermentation.

Consequently, this study was undertaken to determine factors involved in starting and maintaining generators with A. aceti in mineral media; and the major factors which influenced the operation of generators. In addition studies were made to determine some of the factors affecting the oxidation of various carbon sources by A. aceti grown in mineral media.

REVIEW OF LITERATURE

NUTRITION OF ACETIC ACID BACTERIA

Pasteur (1868) noted that Mycoderma aceti (Acetobacter aceti) could grow in a mineral salts medium utilizing the ammonium ion as the sole nitrogen source only if glucose or acetate were added in conjunction with ethanol as a carbon source. Hoyer (1898) and Beijerinck (1898) confirmed these observations. However, Frateur (1950) reported that a phosphate buffer would replace the acetate or glucose requirement so that A. aceti could utilize ethanol as the sole carbon source. This medium is known as modified Hoyer's mineral medium. The same author noted that Acetobacter peroxidans and Acetobacter lovaniense would, also, grow in this medium.

Rao and Stokes (1953a) found that the ability to utilize inorganic nitrogen is not as rare among species of Acetobacter as was previously considered. Many strains of Acetobacter suboxydans and Acetobacter melanogenum possessed this ability under two conditions: (a) the presence of growth factors and (b) the presence of appropriate sources of carbon and energy. A. suboxydans required pantothenic, nicotinic, and p-aminobenzoic acids (PABA), while A. melanogenum required the above growth factors and thiamine. The organisms could utilize either glucose, arabinose, mannitol, or sorbitol served as sources of carbon. Ethanol, pyruvate and lactate could be used as carbon sources only if combined with hydrolyzed casein and an unknown growth factor(s) found in yeast autolysate, tryptone and other complex biological materials. They (1953b)

later reported the unknown growth factor(s) to be reducing sugars or substances related to them.

Studying seven strains of acetic acid bacteria, Rainbow and Mitson (1953) found that Acetobacter acidum-mucosum, Acetobacter mobile, and A. suboxydans could utilize ammonium nitrogen as the sole nitrogen source when glucose and lactate were present as carbon sources. Kaushal and Walker (1951) found that Acetobacter pasteurianum, Acetobacter kutzingianum, and Acetobacter acetigenum would utilize ammonium sulfate as the nitrogen source with ethanol and glucose as carbon sources. Frateur (1950) considered the first two organisms mentioned above as varieties of Acetobacter rancens which he found was unable to grow under such conditions.

In all, ten species of Acetobacter were listed in the literature as having been able to utilize inorganic nitrogen under proper conditions.

Vitamin and Amino acid requirements of various Acetobacter species - A. suboxydans has been suggested for the assay of PABA by Lampen, Unterkofler, and Peterson (1942). This organism required PABA for growth in a basal medium. Karabinos and Dicken (1944) reported that nicotinic acid isolated from milk was essential for the growth of this organism. Unterkofler, Bantz, and Peterson (1943) found that this organism could synthesize riboflavin. In addition, they noted that pantothenic acid or one of its derivatives, alpha-hydroxy-beta-dimethyl-gamma-butyrolactone, PABA, and nicotinic acid were required for the growth of A. suboxydans. According to Stokes and Larson (1945), A. suboxydans required valine, isoleucine, alanine, histidine and cystine

or methionine for growth. Growth occurred at suboptimum levels of these amino acids with the addition of ammonium sulfate.

Litsky, Esslen, Tepper, and Miller (1953) reported that PABA was the only vitamin required for the growth of Acetobacter xylinum. Tepper and Litsky (1953) were able to maintain five serial subcultures of two strains of this organism in a medium containing mineral salts as essential vitamins, and a combination of isoleucine, valine and alanine.

Foda and Vaughn (1953) found six cultures of A. melanogenum to require pantothenic acid, PABA, nicotinic acid, and thiamine; but they did not require amino acids. One culture each of Acetobacter oxydans, and A. rancens required pantothenic acid, PABA, nicotinic acid, and valine, isoleucine, alanine, cystine, histidine, and proline. In addition, A. rancens required either glutamic or aspartic acids for best growth.

Rainbow and Mitson (1953) found that A. suboxydans, Acetobacter capsulatum, Acetobacter gludonicum, Acetobacter turbidans, and Acetobacter viscosum required nicotinate, pantothenate, and p-aminobenzoate Acid hydrolyzed casein is a good source of nitrogen. Ammonia could not be utilized as the sole nitrogen source.

GLUCOSE METABOLISM OF THE PSEUDOMONADACEAE WITH SPECIAL REFERENCE TO ACETOBACTER

It has been known for some time that Acetobacter and other pseudomonads could oxidize glucose with the accumulation of various products

of partial oxidation of glucose. Butlin (1936) reported the oxidation of glucose by A. suboxydans with the accumulation of gluconate. Kluyver and Boezaardt (1938) reported that A. suboxydans produced 2-ketogluconate. Bernhauer and Knobloch (1938), and Bernhauer and Riedl-Tumova (1950) reported that strains of Acetobacter would oxidize glucose to 2- and 5-ketogluconate. Kulka and Walker (1954) found that strains of Acetobacter could accumulate ketogluconate or 2,5-diketogluconate in the medium. Working with cell free extracts of Pseudomonas aeruginosa, Claridge and Werkman (1953) noted the oxidation of glucose to gluconate and 2-ketogluconate.

A great deal of interest arose in respect to the mechanism of these oxidations, and in the metabolic pathway involved in the utilization of glucose by members of this group of bacteria. Wood and Schwerdt (1953) found that Pseudomonas fluorescens would oxidize glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, and 6-phosphogluconate. Stokes and Campbell (1951) found that dried cells of Ps. aeruginosa would oxidize glucose or gluconate to 2-ketogluconate without involving phosphorylation. Wood and Schwerdt (1945) reported the oxidation of glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, and 6-phosphogluconate with the evolution of CO_2 by cell-free extracts of Ps. fluorescens. In addition, they separated the two enzyme systems, and found that the oxidation of glucose proceeded to 2-ketogluconate without phosphorylation, confirming Stokes and Campbell's (1951) finding. Koepsell (1950) found that Ps. fluorescens oxidized 2-ketogluconate to pyruvate, and

Warburton, Eagles and Campbell (1951) found the same to be true for Ps. aeruginosa. Working with resting cells of Pseudomonas saccharophila, Entner and Duodoroff (1952) observed the oxidation of 6-phosphogluconate to pyruvate and triose phosphate under anaerobic conditions. They postulated the formation of an intermediate, 2-keto-3-deoxy-6-phosphogluconate. MacGee and Doudoroff (1954) confirmed the oxidation of 6-phosphogluconate to pyruvate and triose phosphate and isolated an intermediate which was identified as 2-keto-3-deoxy-6-phosphogluconate dehydrase from extracts of Ps. fluorescens. Kovachevich and Wood (1955a) found that this enzyme requires glutathione and ferrous ions for activity. The same authors (1955b), in a subsequent study, found the above enzyme in Acetobacter. Also, they isolated another enzyme, an aldolase, which splits 2-keto-3-deoxy-6-phosphogluconate to pyruvate and D-glyceraldehyde-3-phosphate. This enzyme was found in Acetobacter among other species.

Recently, Rao (1955) did a considerable amount of work on the pyruvate and acetate metabolism of A. aceti and A. suboxydans. He found that the dominant route of pyruvate oxidation in A. suboxydans was through the aldehyde to acetate, whereas A. aceti appeared to form acetate from pyruvate by means of an oxidative decarboxylation. He also found that no tricarboxylic acid cycle (TCAC) intermediates would be oxidized by resting cell suspensions of A. aceti. However, cell free extracts would rapidly oxidize TCAC intermediates. This he attributed to a cell wall permeability barrier. In addition he found that A. aceti had all of the TCAC enzymes, whereas A. suboxydans

possessed only detectable amounts of aconitase and fumarase. He noted that both species had TPN linked acetaldehyde dehydrogenases. In addition, A. aceti had a DPN-specific dehydrogenase. Aldehyde dehydrogenases were not CoA dependent or acyl generating in either species.

King and Cheldelin (1954) working with A. aceti, and Lutwak-Mann (1938) working with A. suboxydans found that the ethanol dehydrogenases of the two species were DPN linked.

OPERATION OF VINEGAR GENERATORS

Unfortunately, the literature is lacking in information pertinent to the operation of vinegar generators. Much of the information available is outdated, and no information is available relative to the physiology of the Acetobacter in generators.

Both Brannt (1914) and Vaughn (1954) pointed out that air flow and pumping rate were important in the operation of a generator. Apparently, both the rate of acetification and the temperature of the generators could be controlled by regulating these two factors.

Brannt (1914) noted four degrees of activity to be found in generators: (a) normal oxidation which occurred when ethanol was converted to acetate, (b) underoxidation which occurred when there was an insufficient air supply or when ethanol concentration was too high, (c) overoxidation which occurred when acetate which had been produced was oxidized and which could be controlled by the addition of ethanol, and (d) superoxidation which occurred when there was an increase in the ethanol concentration in the circulating liquor along with a rise

in temperature of the generator. A musty odor was evident when the latter condition existed. The author claimed that the superoxidation was caused by the incorporation of "too rich nutrients" in the medium; and concluded that this condition often resulted from the use of fresh cider in generators. Brannt (1914) also indicated that in most cases of under- and superoxidation, the only thing that could be done was to restart the generator.

Brannt (1914) noted that Acetobacter cultures must be adapted to ethanol stepwise either in the preparation of a starting culture or in the use of higher concentrations of ethanol in the generators. He stated that ethanol should not be increased more than 2 percent by volume at a time.

Vaughn (1954) stated that cider and wine vinegar were more easily produced in the recirculating generator than distilled vinegar; also, it was easier to seed a cider or wine generator than a distilled vinegar generator. He attributed this to distilled vinegar stock being a mixture of synthetic materials containing no natural nutritive substances as does cider or wine. Thus, it was necessary to add organic and inorganic sources of nitrogen, phosphorous, etc. Most producers rely upon commercially available "bacteria food" which is added in given amounts to diluted ethanol, rather than develop their own formulations.

EFFECT OF THE VINEGAR EEL ON ACETIC FERMENTATIONS

Due to the everpresence of the vinegar eel, Turbatrix aceti var. aceti, in vinegar manufacture, it has been the subject of many studies.

Brannt (1914) felt that its presence was not desired under any conditions. Peters (1928), Potts (1910), Wyant (1919), and Zimmerman (1921) all indicated that the eel impared vinegar production by destroying the film of acetic acid bacteria. However, in none of the cases has it been proven that the eel actually harmed the production capacity of acetic acid bacteria.

Wustenfeld (1930) took the opposite view by pointing out that the eel feeds mostly on available organic material which, in vinegar manufacture, is predominantly acetic acid bacteria. He stated that in many cases it was impossible to operate generators unless they are present. He felt that the eel's metabolites might function as nutrients for the bacteria. Zalkan and Fabian (1953), working with A. aceti, demonstrated that there was a higher acid production and greater efficiencies of conversion of ethanol to acetic acid in generators containing eels than in those without eels.

EXPERIMENTAL METHODS

Organism: Two strains of Acetobacter aceti designated as Nos. 578 and 581 were used. The cultures were supplied by Dr. W. C. Haynes of the Northern Utilization Research Branch of the United States Department of Agriculture, Peoria, Illinois.

Medium: Two mineral media were used: a) the mineral salts mixture described by Hennenberg (HMM) (1926) -- 3.0 g $(NH_4)_4SO_4$, 3.0 g KH_2PO_4, 2.0 g $MgSO_4$ per liter of water; and b) the mineral salts mixture described by Frateur (FMM) (1950) -- 1.0 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.9 g KH_2PO_4, 0.25 g $MgSO_4$ per liter of water. To each of the above was added an appropriate carbon source(s). No appreciable difference should be noted in the growth of either strain of A. aceti in the two media.

Ethanol, denatured by the addition of ethyl acetate (United States Industrial Chemicals Company 35-A) was used unless otherwise specified.

THE EFFECT OF THE CARBON SOURCE ON THE GROWTH OF ACETOBACTER ACETI IN MINERAL MEDIUM

The effect of various carbon sources on growth of A. aceti was tested in the following manner. Thirty ml of medium in a 125 ml Erlenmeyer flask was used for this and subsequent studies. HMM was used as the basal medium and the following carbon sources tested:

a) 2 percent dextrose

b) 0.5 percent acetic acid adjusted to pH 6.0 (acetate).

c) 2 percent ethanol

d) 2 percent dextrose and 0.5 percent acetate

e) 2 percent dextrose and 2 percent ethanol.

To test whether A. aceti requires vitamins or amino acids in a mineral medium with acetate as the carbon source, basal medium plus 0.5 percent acetate was supplemented with 0.5 percent Difco casamino acids, 0.5 percent Difco yeast extract, or a mixture of 9 B-complex vitamins. The vitamins and their concentrations (ug/ml are given as follows: DL Ca pantothenate, 40; nicotinamide, 20; p-aminobenzoic acid, 20; pyridoxine HCl, 40; riboflavin, 40; thiamine HCl, 20; biotin, 0.5; and i-inositol, 20.

The two strains of A. aceti were maintained in HMM containing 2 percent dextrose, 2 percent acetic acid, and 5 percent ethanol for a number of sub-cultures. An inoculum was prepared from a 24-hr culture which had been grown at 30 C in 20 ml of medium contained in a 125 ml Erlenmeyer flask. The culture was washed three times with sterile distilled water and shaken with glass beads in 25 ml of sterile distilled water. The latter procedure was necessary to minimize the amount of "carry-over" of nutrients occluded in the slime produced by these organisms. A volume of 0.25 ml was used to inoculate each test flask. A series of flasks containing various carbon sources was inoculated, incubated at 30 C, and observed for growth response.

THE VITAMIN AND AMINO ACID NUTRITION OF ACETOBACTER ACETI IN MINERAL MEDIUM

It is known that many non-exacting organisms are stimulated by the presence of various vitamins and amino acids included in the growth medium. Despite the fact that A. aceti will grow in dextrose plus mineral salts medium, it was necessary to determine whether its growth could be stimulated by vitamins or amino acids. HMM containing 2 percent dextrose was used as the basal medium. The vitamin stimulation of both strains of A. aceti was studied on one hand by excluding any one of the nine B vitamins in various sets of media and, on the other, by supplementing the basal medium with individual vitamins. Vitamins and concentrations employed were those described previously. One-half percent casamino acids fortified by the addition of 20ug per 100 ml of L-tryptophane and L-cysteine was used as the amino acid source.

Since A. aceti is a film former and the formation of this film appears to follow a definite pattern in the life cycle of standing cultures, the time required for the first formation of a film on the surface of the medium was considered sufficient for determining growth response. In addition, titratable acidity produced after a given period of time was determined in the experiment with single vitamins.

MANOMETRIC STUDIES OF THE OXIDATION OF VARIOUS CARBON SOURCES BY ACETOBACTER ACETI

The manometric studies of the oxidation of various carbon sources by A. aceti grown in mineral medium was carried out at 30 C using the

standard Warburg technique (Umbreit, Burris and Stauffer, 1951). Cell suspensions of both strains of A. aceti were prepared in the following manner. An inoculum was prepared by growing cells in 100 ml of FMM with 0.1 percent dextrose, 0.1 percent yeast extract, and 5 percent ethanol contained in a 250 ml Erlenmeyer flask. After 48 - 72 hours incubation, the contents of the inoculum flask were transferred to a Waring blendor and mixed. The homogenized culture was transferred to one liter of fresh medium, and approximately 10 ml portions were poured into sterile Petri dishes. These were incubated at 30 C for 36 hours. The contents of the Petri dishes were then transferred to a Waring blendor, mixed for one-half minute, and strained through several layers of cheese-cloth. The suspension formed was concentrated by centrifugation and washed four times in distilled water. The washed cells were suspended in 0.075 M phthalate buffer at pH 5.0 containing 100 ppm $MgSO_4$ and stored in the frozen state until used.

STUDIES OF PILOT-PLANT VINEGAR GENERATORS

Four laboratory pilot-plant vinegar generators described by Zalkan and Fabian (1953) were used throughout this study. They consisted of glass towers approximately 15 cm O.D. by 100 cm in height, with provision for recirculation and forced aeration. Each generator was filled with approximately one bushel of beechwood shavings, washed 30 times with hot distilled water and five times with 5 percent acetic acid. The final acid wash was circulated until the time of inoculation.

One liter of a 48-hour culture of strain 581 in FMM with 1.0 percent dextrose, 2 percent acetic acid and 5 percent ethanol, and one

liter of fresh medium, were added to each generator. The air flow was adjusted to approximately 400 ml per minute and the circulation rate was set at 50 ml per minute. The air flow rate was regulated with a needle valve and measured with either a rotameter or a permanent head loss flowmeter which had been calibrated in ml. of air per minute. The operation of the generator was studies as a function of dextrose and ethanol concentrations, and the rate of air flow.

When recharging the generators, they were allowed to drain for 15 to 30 minutes and were then filled with 2 liters of fresh medium. Initial observations were made 10 - 12 hours after recharging. Twenty-five ml samples were drained from the generators at various time intervals and studies were made of the dextrose, ethanol, and acetic acid concentrations. Dextrose was determined colorimetrically as reducing sugar and ethanol was determined by dichromate oxidation (Neish, 1952). Total acid was titrated with 0.1 N NaOH and calculated as percent acetic acid.

The effect of the vinegar eel on the operation of vinegar generators: In commercial operation, it is practically impossible to keep vinegar eels out of vinegar generators. The eels appear to act as scavengers on acetic acid bacteria and much debate has transpired concerning the beneficial or detrimental effects of the eel on the acetification process. Consequently, experimental generators were deliberately infected with these nematodes in the following manner. The eels in one-half gallon of commercial vinegar supplied by Libby, MacNeil and Libby Co., Blue Island, Illinois, were concentrated by filtering

through a filter paper in a Buchner funnel. They were then washed five times with sterile saline and suspended for 15 minutes in a $HgCl_2$ solution described by White (1931); washed five more times and suspended in 100 ml of sterile saline. Fifty ml. of the suspension of eels were inoculated into each of two generators. Previous studies indicated that the above contact time in White's solution was sufficient to inhibit all organisms that would grow in nutrient agar acidified to 5 percent acetic acid, without causing any apparent harm to the eels.

RESULTS

THE EFFECT OF CARBON SOURCE ON THE GROWTH OF *ACETOBACTER ACETI* IN MINERAL MEDIUM

A great deal of difficulty was encountered with initial attempts to grow both strains of *Acetobacter aceti* in mineral medium. Eventually, the organism grew in mineral medium containing dextrose as a carbon source; however, it could not be grown if the dextrose were replaced with ethanol and/or acetate. Attempts were made to remedy this situation by varying concentrations of carbon source(s), pH, *etc.*, but the organism would not grow with ethanol and/or acetate alone. It was observed that *A. aceti* grown in mineral medium with dextrose would continue growing in ethanol or acetate under mineral conditions if the entire flask contents were transferred. Therefore, it was suspected that the organisms inability to grow in the presence of ethanol and/or acetate was due to carbon source. Thus a study of the effect of carbon source on the growth of *A. aceti* in mineral-salts-medium was made.

The results of the study of the growth of two strains of *Acetobacter aceti* in mineral medium with various carbon sources is presented in Table 1. The organisms grew with dextrose but not with ethanol or acetate. Growth in a medium containing ethanol or acetate occurred upon the addition of dextrose.

Subsequent experience with *A. aceti* pointed out that dextrose must always be included in a mineral-salts-ethanol medium for growth and acetic acid production. Apparently, only traces of dextrose are necessary, since 0.01 percent has been observed to satisfy this requirement.

TABLE 1

Growth of A. aceti in HMM with various carbon sources

Carbon Source	Strain No. 578	Strain No. 581
Dextrose (2%)	+*	+
Ethanol (2% by vol.)	-	-
Acetate (0.5%)	-	-
Dextrose (2%) and ethanol (2% by vol.)	+	+
Dextrose (2%) and acetate (0.5%)	+	+

* + = growth, - = no growth.

TABLE 2

Growth of A. aceti in HMM plus 0.5 percent acetic acid (pH 6.0) with vitamin and amino acid supplements.

Supplement	Strain No. 578	Strain No. 581
Yeast extract (0.5%)	+*	+
9 B-complex vitamins**	-	-
Casamino acids (0.5%)	+ sl.	+ sl.

* + = growth, - = no growth, + sl. = slight growth.
** See text (p. 12) for vitamins and concentrations used.

Pasteur (1868), Hoyer (1898), and Beijerink (1898) reported that A. aceti could grow in a mineral-salts-ethanol medium if acetate or acetic acid were added. Since our results did not confirm this finding it was necessary to determine if the organism would grow if acetate were supplemented with various nutritional factors. Table 2 presents the results of the study of the growth of two strains of A. aceti in

mineral-salts-acetate medium supplemented with yeast extract, vitamins, and amino acids. Growth occurred in the presence of yeast extract, but there was no growth with the 9 B-complex vitamins. Slight growth was evident in the presence of Casamino acids.

It is possible that the factor in yeast extract may be some source of carbon which is available to the organism. This would parallel the finding of Rao and Stokes (1953b) with Acetobacter suboxydans. It is possible that the slight growth observed in the presence of amino acids and acetate could be attributed to the deamination of amino acid(s) such as alanine. The pseudomonads are generally quite capable of de- and transaminations, and it is assumed that members of the Acetobacter genus are no exceptions.

THE EFFECT OF VITAMINS AND AMINO ACIDS ON THE RATE OF GROWTH AND ACID PRODUCTION BY ACETOBACTER ACETI IN MINERAL MEDIUM PLUS DEXTROSE

Although A. aceti grew in mineral-salts-dextrose medium, the possibility existed that it would be stimulated by the addition of various vitamins and/or amino acids. The following experiments were undertaken to determine if this did occur. Table 3 shows the results of the study of growth response of two strains of A. aceti in mineral medium containing dextrose fortified with vitamins and/or amino acids. Vitamins or vitamins and amino acids were found to stimulate the growth of the organisms, but amino acids alone were not effective.

It was of interest to know whether combinations of particular vitamins caused the observed stimulation, or whether it was the result of individual vitamins. The results of the study of the growth

TABLE 3

Time required for film formation of *A. aceti* in HMM plus 2 percent dextrose with vitamin and amino acid supplements.

Supplement	Days required for film formation	
	Strain no. 578	Strain no. 581
None	2	4
9 B-complex vitamins*	1	2
Casamino acids (0.5%)	2	4
9 B-complex vitamins* plus Casamino acids (0.5%)	1	2

* See text (p.12) for vitamins and concentrations used.

TABLE 4

Effect of the ommission of various vitamins from the vitamin supplement on the time required for film formation by *A. aceti* in HMM plus 2 percent dextrose.

Vitamin omitted	Days required for film formation	
	Strain No. 578	Strain no. 581
None	1	2
Pantothenate	1	2
folic acid	1	2
nicotinamide	1	2
p-aminobenzoate	1	2
pyridoxine	1	2
riboflavin	1	2
thiamine	1	2
biotin	1	2
inositol	1	2

response of A. aceti in the presence of various mixtures of B-vitamins is presented in Table 4. The growth response was the same in all cases for each strain. However, the stimulatory effect of the addition of single vitamins on the acid production and growth of A. aceti, presented in Table 5, showed that growth was more rapid in the presence of Ca pantothenate, folic acid, thiamine and biotin. There was no appreciable effect of these four vitamins on the amount of acid produced by strain 578; however, acid production by strain 581 was stimulated.

These data indicate that the growth of A. aceti is stimulated by single vitamins, and not by a combination of particular cofactors; in addition no vitamin requirement exists.

TABLE 5

Effect of vitamins on the rate of film formation and acid production by *A. aceti* in HMM plus 2 percent dextrose

Supplement	Hours required for film formation		Mls of 0.1 N NaOH	
	Strain no. 578	Strain no. 581	Strain no. 578*	Strain no. 581**
None	24	48	2.17	1.67
pantothenate	12	36	1.92	3.62
folic acid	12	36	2.17	4.10
nicotinamide	24	48	1.80	1.67
p-aminobenzoate	24	48	1.90	1.47
pyridoxine	24	48	1.87	1.55
riboflavin	24	48	1.82	1.50
thiamine	12	36	1.97	3.90
biotin	12	36	1.85	3.40
inositol	24	48	1.85	1.52
all vitamins	12	36	1.97	4.10

* Average of duplicate 10 ml samples titrated to pH 7.0 after 36 hours incubation.

** Average of duplicate 10 ml samples titrated to pH 7.0 after 72 hours incubation.

THE OXIDATION OF VARIOUS CARBON SOURCES BY *ACETOBACTER ACETI*

The information available on the oxidative characteristics of the *Acetobacter* has been obtained using suspensions or extracts of cells grown in enriched medium with either dextrose or glycerol as a carbon source. Since the primary purpose of this study was to obtain information concerning *Acetobacter aceti* grown in mineral medium with the utilization of ethanol as an energy source, it was necessary to study the oxidative characteristics of this organism grown under such conditions. In Table 6 and Figure 1 are presented the results of the study of the oxidation of various carbon sources by *A. aceti* grown in a mineral medium containing 0.1 percent yeast extract, 0.1 percent dextrose, and 5 percent ethanol. No oxidation of dextrose, gluconate, pyruvate, or acetate was evident with either strain. Both strains oxidized ethanol, using one mole of oxygen per mole of ethanol. This would indicate the conversion of ethanol to acetate. Further studies were made combining ethanol and dextrose; but in each case the oxygen uptake corresponded to the oxidation of ethanol to acetate.

Since the growth of *A. aceti* with dextrose was stimulated by the addition of certain vitamins, dextrose and ethanol were combined with 1 ug of pantothenate, folic acid, thiamine and 1 mug of biotin. Table 7 and Figure 2 present the results of this study. No oxygen uptake was evident with dextrose and vitamins. The oxygen uptake with ethanol and vitamins corresponds to the oxidation to acetate.

It had been demonstrated earlier in this work that both strains grew in a mineral medium containing either dextrose and ethanol, or

TABLE 6

Oxygen uptake by *A. aceti* with various carbon sources.

Substrate	Strain No.	Oxygen uptake (ul) at various times (min.) 0	5	10	15	20	25	30	40
Glucose (1mM)	578	0	--	0	--	0	--	--	0
	581	0	0	0	0	0	0	--	--
Gluconate (1uM)	578	0	--	0	--	0	0	0	0
	581	0	0	0	0	0	0	--	--
Pyruvate (1uM)	578	0	--	0	--	0	--	0	0
	581	0	0	0	0	0	0	--	--
Acetate (5uM)	578	0	--	0	--	0	--	0	0
	581	0	0	0	0	0	0	--	--
Ethanol (5uM)	578	0	--	80	--	106	--	106	106
	581	0	58	91	105	113	113	--	--

TABLE 7

Oxygen uptake by *A. aceti* strain 581 with dextrose and ethanol in the presence of vitamins.*

Substrate	Oxygen uptake (ul) at various times (min.) 0	10	20	30	35	40
Glucose (1uM)	0	0	0	0	0	0
Ethanol (10uM)	0	103	186	210	213	213

* For vitamins and concentrations used see text (p. 23).

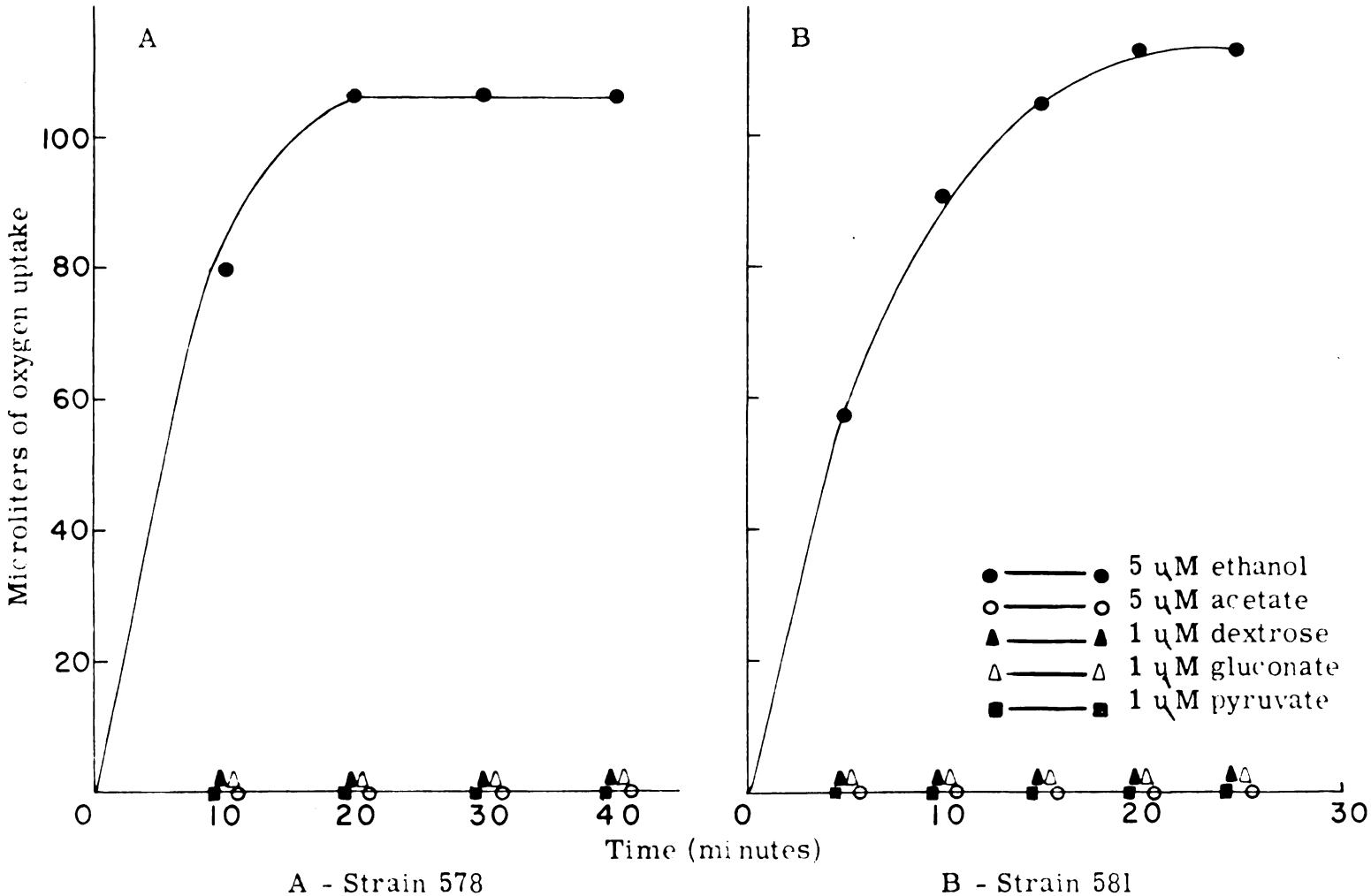

Figure 1. Oxygen uptake by A. aceti with various carbon sources.

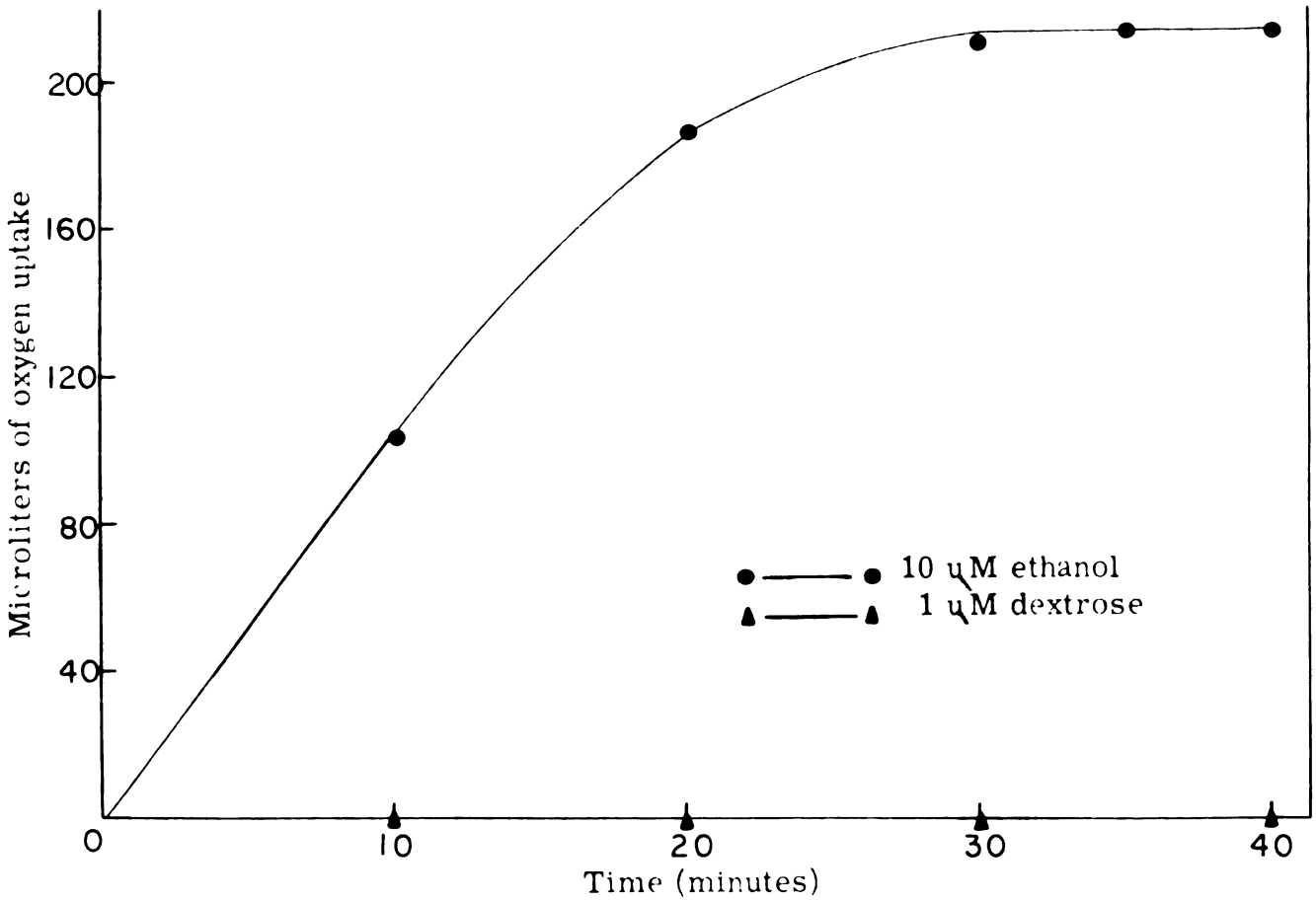

Figure 2. Oxygen uptake by *A. aceti* strain 581 with dextrose and ethanol in the presence of vitamins.

dextrose and acetate. Therefore, it was evident that they could utilize dextrose for energy if necessary. Consequently, it was necessary to determine if A. aceti grown in a dextrose containing medium would oxidize dextrose and acetate. A suspension of one strain was prepared as described previously with the exception that cells which had been transferred in mineral medium plus dextrose were grown in shake flasks for 12 hours at 30 C. The medium used was FMM containing 1 percent yeast extract and 1 percent dextrose.

The oxidation of dextrose, ethanol and acetate by strain 578 is presented in Table 8 and Figure 3. The uptake was 1.5, 3.0 and 2.0 moles of oxygen per mole of dextrose, ethanol, and acetate respectively. This corresponds to a partial oxidation of dextrose, and complete oxidation of both ethanol and acetate.

TABLE 8

Oxygen uptake with various carbon sources by A. aceti strain 578 grown in enriched medium

Substrate	Oxygen uptake (ul) at various times (min)							
	0	10	20	30	40	50	60	70
Glucose (1uM) Trial 1	0	3	12	19	25	29	36	37
Trial 2	0	1	12	17	24	28	34	36
Ethanol (1uM)	0	7	16	25	37	46	54	56
Acetate (1uM)	0	9	18	28	40	45	46	47

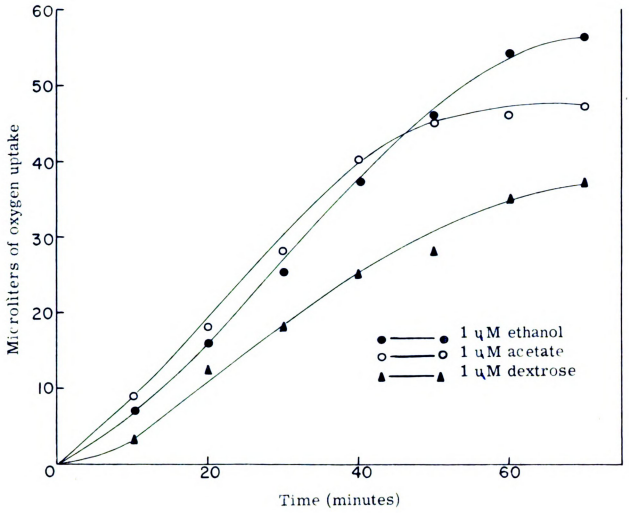

Figure 3. Oxygen uptake with various carbon sources by A. aceti strain 578 grown in enriched medium.

STUDIES OF PILOT - PLANT VINEGAR GENERATORS

The effect of dextrose concentration on the operation of vinegar generators - The generators were inoculated with a mineral medium containing 1 percent dextrose as previously described. According to Vaughn (1954), acid production subsequent to inoculation in generators will generally initiate in 7 - 10 days. In the present experiment, acid production began after 72 hours, continued for approximately 5 days, and then stopped. It was thought that the dextrose concentration was possibly too high, therefore the generators were recharged with medium containing no dextrose. Table 9 and Figure 4 present changes in total acid, dextrose, and ethanol concentrations in generators after this modification. The acid concentration in the four generators did not change. There appeared to be a tendency for a slight decrease in the ethanol concentration followed by a slight rise. This may be due to a sampling error. The dextrose concentration increased, eventually reaching a constant level. The reason for the increase in dextrose is unknown at the present time. It may have been due to the sloughing off and/or hydrolysis of the slime present in the generators, or it may have been due to some as yet unexplained factor in the organism's metabolism. It has been reported (anonymous, 1955) that optimum acid production in experimental generators inoculated with Acetobacter xylinum was achieved if the dextrose concentration was maintained between 0.05 - 0.07 percent. The activity of the generators was restored on reducing the dextrose concentration to this level by dilution. In subsequent experiments, fresh medium contained 0.05 percent dextrose.

TABLE 9

Changes occurring in generators with high initial dextrose concentrations

Generator No.		Concentrations at various intervals - Days					
		0	1	2	3	4	7
1	% acid*	3.70	3.25	3.06	3.30	3.06	2.97
	% dextrose**	0.41	0.75	0.45	1.00	1.00	1.00
	% ethanol***	4.68	4.30	4.34	4.08	4.44	5.22
2	% acid	3.90	4.16	4.17	4.26	4.02	3.90
	% dextrose	0.13	0.21	0.21	0.33	0.46	0.36
	% ethanol	6.20	5.84	5.38	4.64	4.58	5.80
3	% Acid	4.38	4.60	4.71	4.59	4.56	4.50
	% dextrose	0.12	0.11	0.17	0.32	0.37	0.40
	% ethanol	5.76	5.16	4.80	4.64	4.88	4.52
4	% acid	4.20	4.42	4.50	4.32	4.50	4.44
	% dextrose	0.11	0.15	0.16	0.36	0.36	0.41
	% ethanol	5.39	4.66	4.52	4.40	4.16	4.71

* % acid (gms/100 ml) as acetic acid.
** Reducing sugar calculated as percent dextrose.
*** Percent ethanol, w/v.

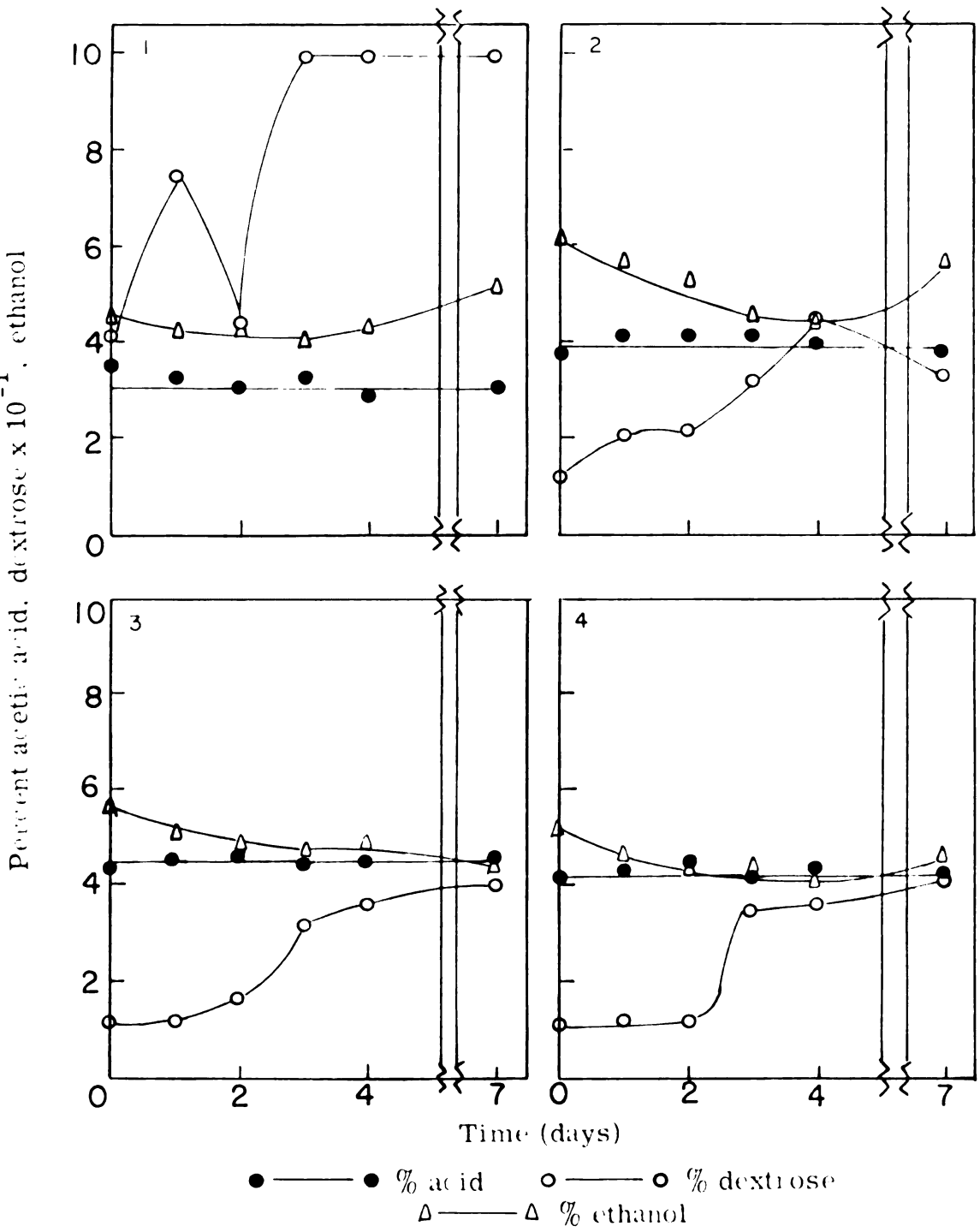

Figure 4. Changes occurring in generators with high initial dextrose concentrations.

Changes occurring in generators with an air flow of 125 ml per minute - An impericism guiding the vinegar industry states that the yield of cider vinegar from generators should be 0.25 gal. of 6 percent vinegar per bushel of shavings per 24 hours (Vinegar Handbook 1919). Since 1 mole of ethanol requires one mole of oxygen for oxidation to the acetate level, it is possible to calculate the air flow rate required. Based on Vaughn's (1954) recommendations, it was calculated that the generators used in this study required 125 ml of air per minute. This figure is based on an oxygen adsorption efficiency of 50 percent. This supply of air proved to be inadequate. Table 10 and Figure 5 show the effect of air supply on the changes in acetic acid, dextrose, and ethanol concentrations in generators. Acid concentration in the four generators did not change. Both dextrose and ethanol concentrations appeared to fluctuate. Here it appears that the organism could not obtain enough oxygen to oxidize the intermediates formed and would alternately take up and return carbon to the circulating liquor. Initially, it was thought that too much ethanol was present but dilution to 1 percent did not remedy the situation. When air flow was increased, the generators began to produce acid within 48 hours.

Changes occurring in generators with an air flow of 400 ml per minute - Table 11 and Figure 6 show changes occurring in acetic acid, dextrose, and ethanol concentrations in generators with ethanol concentrations ranging from about 4 to 6 percent and an air flow of 400 ml per minute. In general, the acid concentration increased at a slow rate and leveled off, despite the appreciable quantities of ethanol

TABLE 10

Changes occurring in generators with an air flow of 125 ml per minute

Generator No.		Concentrations at various intervals - Days						
		0	1	2	3	4	5	6
1	% acid*	1.68	1.65	1.65	1.68	1.68	1.71	1.68
	% dextrose**	.029	.029	.042	.036	.036	.029	.032
	% ethanol***	5.00	5.10	4.97	5.07	4.77	4.95	4.37
2	% acid	1.74	1.68	1.68	1.74	1.70	1.71	1.71
	% dextrose	.032	.031	.040	.035	.038	.030	.036
	% ethanol	5.05	5.10	5.15	5.15	5.72	5.20	4.70
3	% acid	2.25	2.25	2.25	2.30	2.30	2.33	2.33
	% dextrose	.028	.028	.042	.036	.039	.030	.035
	% ethanol	4.52	4.75	4.42	3.90	4.77	4.20	4.47
4	% acid	1.80	1.80	1.77	1.77	1.84	1.83	1.83
	% dextrose	.045	.045	.050	.049	.049	.040	.044
	% ethanol	4.82	4.32	4.57	4.50	4.77	4.35	4.57

* % acid (gms./100 ml) as acetic acid.
** Reducing sugar calculated as percent dextrose.
*** Percent ethanol, w/v

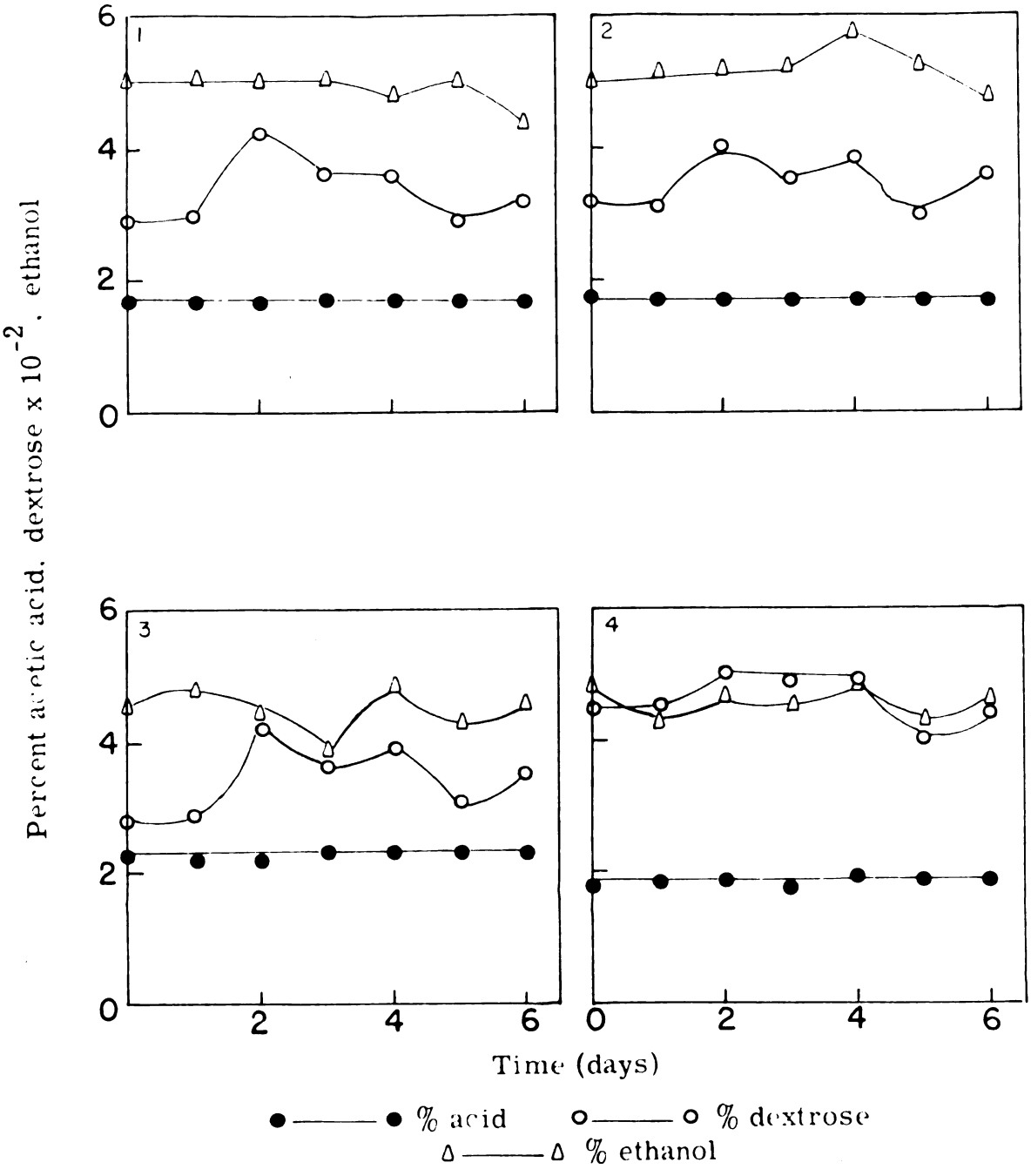

Figure 5. Changes occurring in generators with an air flow of 125 ml per mimute.

TABLE 11

Changes occurring in generators with an air flow of 400 ml per minute

Generator No.		Concentrations at various intervals - Days			
		0	1	2	3
1	% acid*	2.3	2.76	2.82	2.94
	% dextrose**	.041	.034	.030	.030
	% ethanol***	3.85	3.85	1.98	1.22
2	% acid	1.95	2.94	3.48	3.24
	% dextrose	.039	.024	.022	.024
	% ethanol	5.50	4.27	2.15	1.29
3	% acid	1.94	2.64	3.00	3.12
	% dextrose	.034	.019	.015	.015
	% ethanol	6.10	4.87	2.45	1.52
4	% acid	2.16	3.00	3.30	3.24
	% dextrose	.035	.026	.019	.022
	% ethanol	5.35	3.99	2.45	1.29

* % acid (gms./.00 ml) as acetic acid.
** Reducing sugar calculated as percent dextrose.
*** Percent ethanol, w/v.

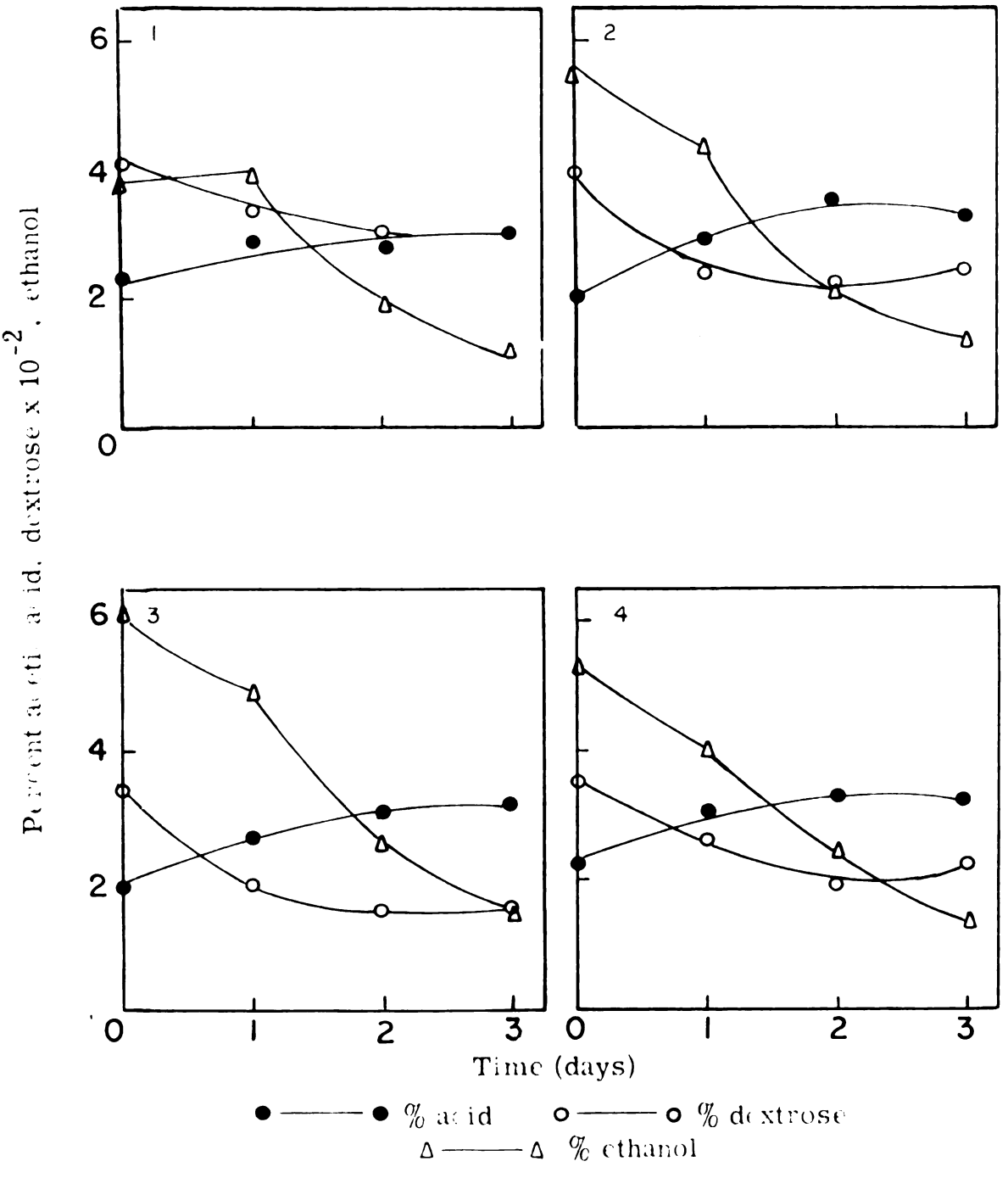

Figure 6. Changes occurring in generators with an air flow of 400 ml per mimute.

still remaining in the generators. The dextrose concentration decreased in all cases, and there appeared to be a lag in the uptake of ethanol. There was considerably more ethanol taken up than can be accounted for by the acid produced. Stochiometrically, 1 gram of ethanol yields 1.26 grams of acetic acid. The ethanol was either lost by evaporation, or was converted to end products other than acetate. Upon dilution of the ethanol to 2 to 3 percent, normal fermentation began. Therefore, the above phenomena appear to have been caused by ethanol concentration.

The effect of vinegar eels on the operation of vinegar generators - Table 12 and Figure 7 show the effect of vinegar eels on the changes in acetic acid, dextrose, and ethanol concentrations in normally functioning generators with an air flow rate of 400 ml per minute. The acid increased at a rapid rate in all cases. Dextrose and ethanol decreased rapidly in all instances, and no significant amounts of ethanol remained in the circulating liquor after 5 days. No measurable effect of the vinegar eel on the acetic acid fermentation could be seen in this experiment. However, there was less slime on the beechwood shavings in the generators infected with eels and they had a "brighter" appearance than in the shavings in the generators free of eels.

In this and in similar experiments it was noted consistently that the decrease in ethanol during a given period of time was less than the corresponding acid increase. Calculated efficiencies of ethanol conversion ranged from 100 - 200 percent. The determinations for acid and alcohol proved to be accurate to the best of the author's knowledge. Therefore, it was thought that the design or use of the generators

TABLE 12

The effect of vinegar eels on the operation of normally functioning vinegar generators.

Generators		Concentration at various intervals - Days							
		0		2		4		5	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
(control)	% acid*	2.82	2.85	4.67	4.37	6.0	5.34	-	5.82
	% dextrose**	.041	.036	.026	.025	.017	.015	-	.012
	% ethanol***	2.13	2.04	1.15	1.02	0.25	0.33	-	0.15
(control)	% acid	2.82	3.72	4.61	4.80	5.70	5.94	-	6.00
	% dextrose	.041	.030	.022	.021	.017	.012	-	.007
	% ethanol	1.89	1.57	1.15	.076	0.41	0.25	-	0.15
(with eels)	% acid	1.98	3.66	4.15	5.05	5.57	6.00	-	5.88
	% dextrose	.037	.035	.020	.015	0.0	.010	-	.007
	% ethanol	2.54	1.47	1.23	0.44	0.82	0	-	0
(with eels)	% acid	3.72	4.62	4.92	5.46	5.85	5.94	-	6.00
	% dextrose	.037	.035	.026	.020	0.0	.012	-	.007
	% ethanol	2.05	1.06	1.06	0.55	0.57	0	-	0

* % acid (gm./100 ml) as acetic acid.
** Reducing sugar calculated as percent dextrose.
*** Percent ethanol w/v.

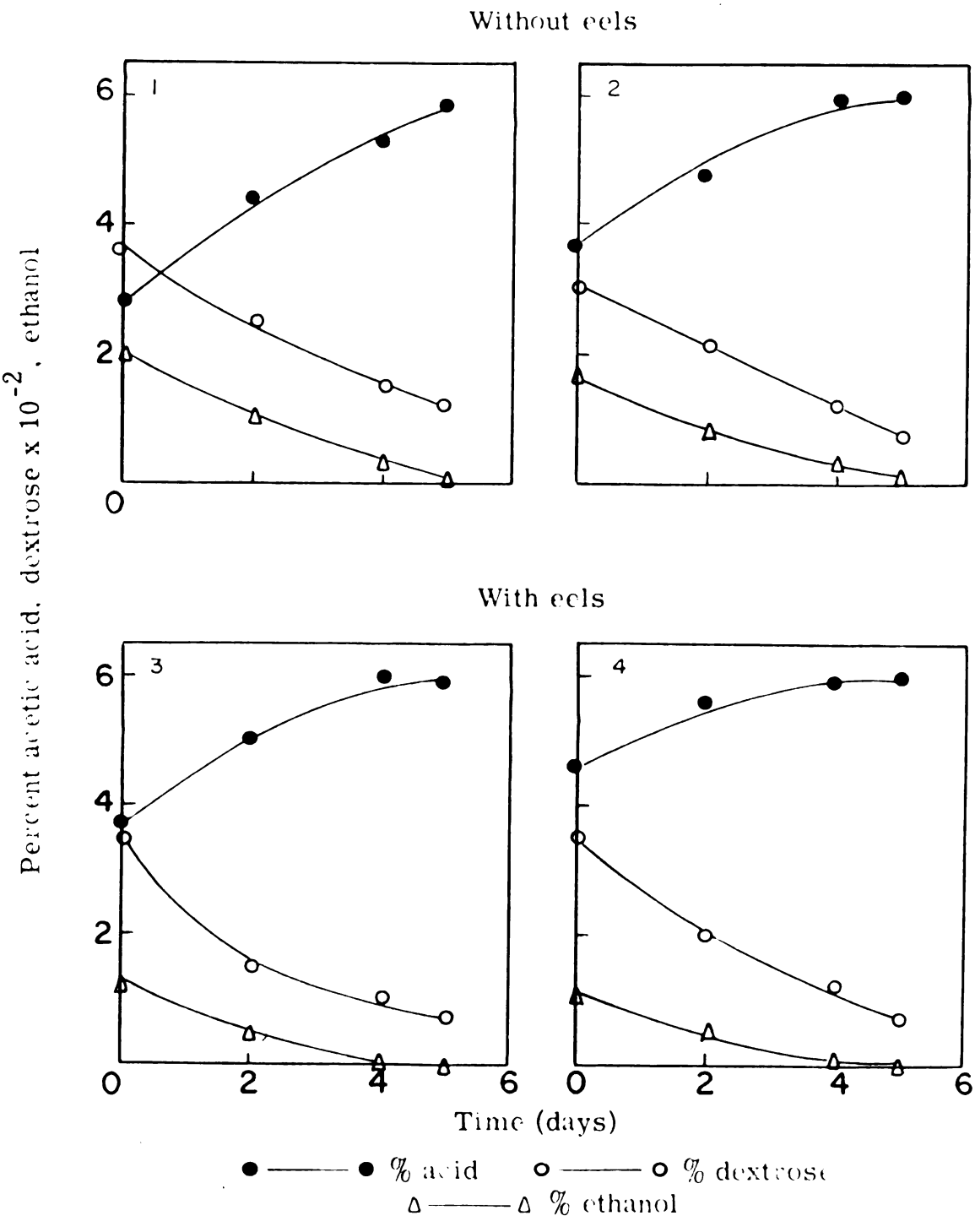

Figure 7. The effect of vinegar eels on the operation of normally functioning vinegar generators.

may have been faulty. Figure 8 shows a drawing of a typical generator used in this study. It was noted that there was a hold-up volume of approximately 1 liter of medium above the air inlet, and it was possible that the air bubbling through this medium might have a stripping effect, resulting in a higher ethanol concentration in the upper portion of the medium than in the area of sampling.

A sample was drained from a generator and another was taken from the area above the air inlet by a hypodermic syringe and needle. The ethanol concentration in the upper area was 3.52 percent and that in the lower area was 2.38 percent. Consequently, the generators should to be modified in future studies by using a larger collection reservoir so that the surface of the alcoholic solution will be below the air inlet.

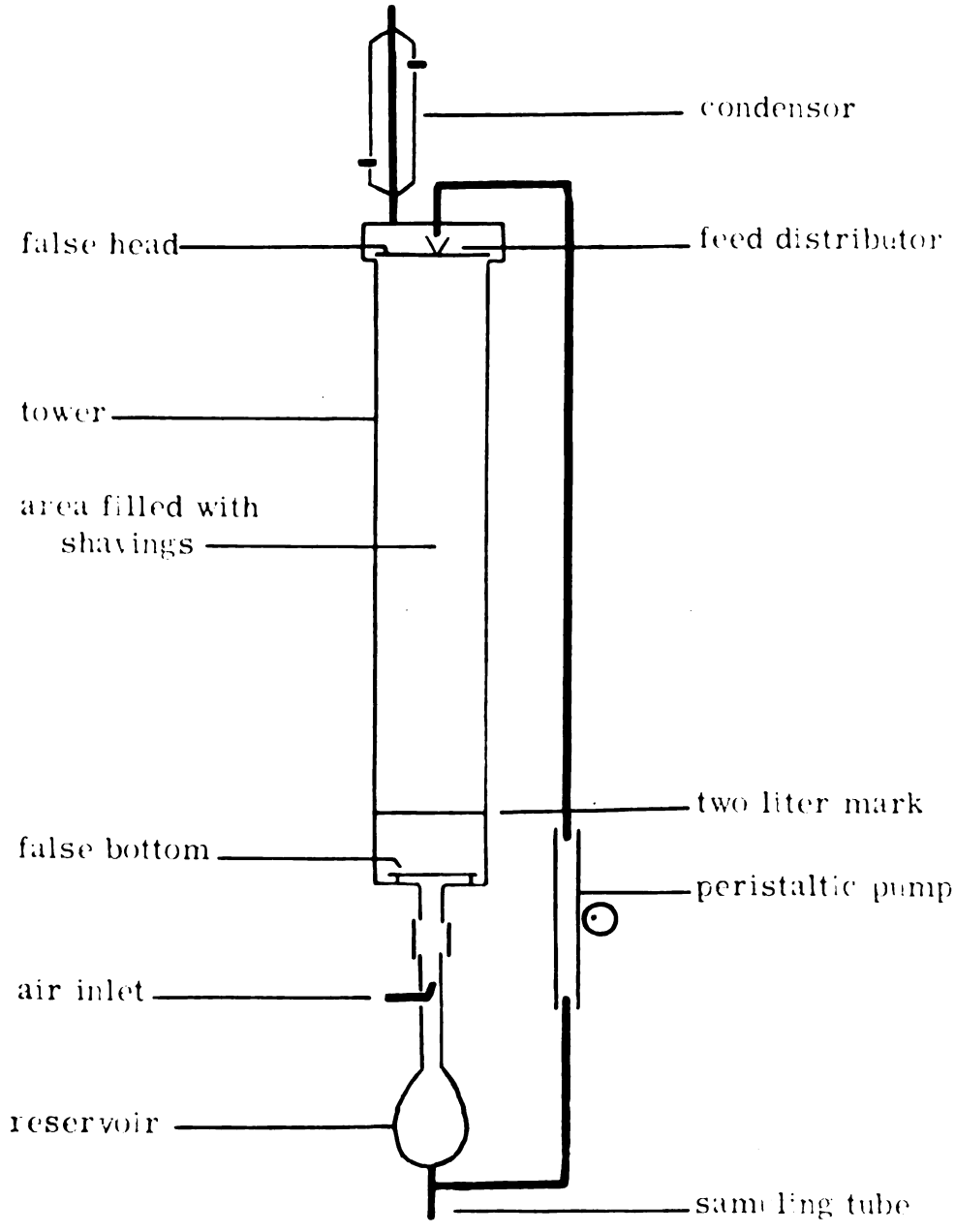

Figure 8. Pilot-plant laboratory vinegar generator.

DISCUSSION

The two strains of Acetobacter aceti studied could not be grown in a mineral medium containing ethanol or acetate unless dextrose was present. Growth would not occur in mineral salts acetate medium to any significant degree even when supplemented with vitamins and amino acids. This finding conflicted with previously reported data (Pasteur, 1868; Beijerink, 1898; Hoyer, 1898) which indicated that A. aceti would grow in a mineral salts medium containing dextrose plus ethanol; and that acetate would replace the dextrose. Neither was it possible to grow the organism in Frateur's medium (1950) in which A. aceti has been reported to grow utilizing ethanol as the sole carbon source. In data which were not reported in this study it was impossible to grow the organism in this medium using cell suspensions which had been maintained in ethanol containing mineral medium for a number of subcultures as the inoculum. The observation of the need for dextrose along with ethanol in mineral medium agrees with the finding of Rao and Stokes (1953b) who found that Acetobacter suboxydans would not grow in a basal medium supplemented with required vitamins and amino acids unless small quantities of dextrose or other reducing sugars were present. The physiology of the acetic acid bacteria pertinent to their growth in and utilization of ethanol is not well understood, and additional work should be done to clarify this issue.

Cell suspensions of A. aceti grown in a mineral medium plus 0.1 percent dextrose, 0.1 percent yeast extract, and 5 percent ethanol would not oxidize dextrose, gluconate, pyruvate, or acetate, and would

only oxidize ethanol to acetate. This is of notable significance since A. aceti has always been considered to be able to convert any available carbon source to carbon dioxide and water. The ability of the cells to oxidize dextrose or acetate was not restored upon the addition of four B-complex vitamins which stimulate A. aceti in dextrose containing mineral medium. However, evidence was obtained which demonstrated that cells of A. aceti grown in mineral medium containing 1 percent dextrose and 1 percent yeast extract would completely oxidize ethanol and acetate, and would take up 1.5 moles of oxygen per mole of glucose. This partial oxidation of glucose would correspond to the formation of 2-, 5-diketogluconate. This may be a step in the formation of slime. Kulka and Walker (1954) have shown that Acetobacter will accumulate this product in the growth medium. In addition Kondo and Takeda (1952), and Koepsell (1950) have shown that the degree of dextrose oxidation is dependent on the nitrogen content of the medium. Koepsell (1950) has also shown that the extent of dextrose oxidation depends upon the iron content of the medium.

These data indicate that A. aceti grown under generator conditions is not a complete oxidizer, and, therefore, may be suitable for the production of grain or distilled vinegar commercially. It is possible that Acetobacter xylinum will behave as does A. aceti in this regard.

It was possible to start and maintain pilot-plant vinegar generators inoculated with A. aceti using a mineral medium as the substrate. The yield of acid from these generators was satisfactory -- approximately 1 percent per day during early stages of the fermentation. The major

factors to be controlled in generators as indicated in this study appeared to be concentrations of dextrose and ethanol, and air supply. The role played by dextrose in the generators is not understood. Apparently, it is not used for energy as previously claimed, however, it is necessary for growth. It has been pointed out by Alexander and Wilson (1954) that air supply is critical for sufficient production of cells of Azotobacter. Hromatka, Ebner, and Csolich (1951) found that Acetobacter in submerged fermentations would die rapidly in the absence of air.

It is the opinion of this author that insufficient air supply, and amounts of dextrose or ethanol in excess of optimum concentrations are all important factors in the physiological activity of the acetic acid bacteria. It appears that an optimum air-flow can be achieved; under which conditions, dextrose and ethanol concentrations can be varied without causing such drastic impairment of the fermentation as observed in this study. However, the maximum air-flow allowed under the present design of the generators used in this study is 400 ml per minute.

No effect of the vinegar eel upon the fermentation rate could be observed in this study. Since the phenomena observed by Zalkan and Fabian (1953) occurred in generators free of dextrose, the eel may supply some essential factor(s) which the acetic acid bacteria derive from dextrose.

SUMMARY AND CONCLUSIONS

Little information is available regarding the acetic fermentation in mineral medium. This study was undertaken to determine factors affecting the growth in and oxidation of ethanol by *Acetobacter aceti* grown in mineral medium.

Two strains of *A. aceti* could be grown in mineral medium containing ethanol only upon the addition of dextrose. Growth did not occur in mineral medium plus acetate on the addition of vitamins, but slight growth occurred on the addition of amino acids. Growth occurred in the presence of yeast extract. The additions of pantothenic acid, folic acid, thiamine, and biotin stimulated the growth of both strains in dextrose mineral salts medium but stimulated acid production by one strain only. The growth of *A. aceti* was not stimulated by the addition of amino acids, and the organism did not demonstrate any vitamin requirements.

Cells of *A. aceti* grown in a mineral medium containing ethanol and dextrose oxidized ethanol to acetate, but would not oxidize dextrose, gluconate, pyruvate, or acetate. However, cells of *A. aceti* grown in medium containing 1 percent dextrose and 1 percent yeast extract completely oxidized ethanol and acetate, and partially oxidized glucose. It is believed that glucose was oxidized to 2,5-diketogluconate, or similar end products. Further, it was postulated that the formation of slime by these bacteria may be a result of this partial oxidation.

Experimental pilot-plant type vinegar generators inoculated with *A. aceti* were started and maintained on a mineral medium. This study

indicated that the major factors to be controlled in the operation of generators were the concentrations of dextrose and ethanol, and the air supply. With the above factors controlled, acid was produced at the rate of one percent per day initially. The vinegar eel appeared to have no effect on the fermentation in normally functioning generators.

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