# FORMATION OF ESTERS BY YEAST

## I. The Production of Ethyl Acetate by Standing Surface Cultures of Hansenula anomala

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It has been known for some time (Hansen, 1891) that organic esters may be produced in small amounts by some microorganisms as by-products in their utilization of organic compounds. A detailed review of the literature to 1922 on aroma producing microorganisms is given by Omelianski (1923). The taxonomy of the yeast genus *Hansenula*, which contains species producing the greatest yields of ester, is discussed by Bedford (1942) and Gray (1949). The early observations on the physiology of ester formation were reviewed by Tabachnick (1950). While our studies of ester formation by yeast, begun in 1946, were in progress, several papers appeared on the physiology of ester formation by *H. anomala*.

Gray (1949), working with standing surface cultures of *H. anomala* (Hansen) in a glucose medium, showed: (1) that ester was produced under conditions of limited aeration from some unknown anaerobically produced intermediate and (2) that the ester was utilized as rapidly as it was produced. Although the ester had been identified by its odor as ethyl acetate (Takahashi and Sato, 1911; Bedford, 1942), Gray was the first to identify it as ethyl acetate. Davies *et al.* (1951) presented additional proof that the ester is ethyl acetate. Peel (1951), using cell suspensions of *H. anomala* in an ethanol, acetic acid medium, found ester formation to be an aerobic process with an optimum pH of 4.5 to 5. In ethanol alone the pH optimum was difficult to define but appeared to range from 2.5 to 3. Peel also showed with growing cells and cell suspensions that the amount of ester formed is too large to be accounted for by the reversal of a simple esterase reaction, indicating a more exergonic mechanism of ester formation.

In the following report on growing cultures of *H. anomala*, some of the observations recorded by Gray (1949), Davies *et al.* (1951), and Peel (1951) are confirmed. These workers identified the ester indirectly; we isolated and purified sufficient ester to determine both physical and chemical properties. In addition, it will be shown that ethyl acetate is not produced directly from glucose but is formed as the result of an aerobic utilization of ethanol accumulated in the fermentation of glucose. The influence of several environmental factors on ester production will be discussed.

### METHODS

*Cultures.* The yeast cultures used for the major portion of this work were *Hansenula anomala* var. *longa* type B (Naegeli) Dekker (culture no. 317 in the Food Technology collection) and *Pichia kluyveri* Bedford (culture no. 7 in the collection). Both these yeasts have a predominantly oxidative metabolism and form a film on the surface of standing liquid cultures.

*Composition of media.* Medium 1—contained $KH_2PO_4$, 1 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 2H_2O$, 1 g; NaCl, 1 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; thiamin hydrochloride, 100 $\mu$g; pyridoxine hydrochloride, 200 $\mu$g; biotin, 2.5 $\mu$g; in 1,000 ml of distilled water. As carbon source glucose or ethanol was used in concentrations given below. This medium, although chemically defined, was not a complete medium for the growth of *H. anomala*, the rate of glucose utilization in medium 2 being considerably more rapid. Medium 1a—contained the same constituents as medium 1 but the growth factors were omitted. Medium 2—had the same salt constituents as medium 1 but the growth factors were replaced by 50 ml of liquid yeast autolysate per liter. Medium 3—Wickerham's nitrogen base medium (Wickerham, 1946).

The media were dispensed in two liter amounts into 2,500 ml narrow necked Fernbach flasks. A double strength solution of the inorganic salt

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constituents was heat sterilized separately from the glucose solution, and both were mixed then aseptically after cooling to approximately 70 C. The ethyl alcohol was sterilized by passage through a bacterial sintered glass filter, and the approximate amount was added to the appropriate basal medium. The requisite amount of growth factor stock solution (20 × strength) was sterilized by filtration before addition to the medium. The yeast autolysate was also sterilized by filtration. The initial pH of all the media used was approximately 4.5.

One per cent inoculum of young actively growing cells was used. In the earlier experiments where the saponification method for ester determination (Amerine, 1944) was used, 100 ml of sample was withdrawn aseptically for analysis, but with the colorimetric method (Hestrin, 1949) 50 ml or less of sample sufficed.

*Analytical methods. pH.* The pH was determined to ±0.05 with a Beckman industrial model glass electrode assembly.

*Total acid.* Ten or 25 ml aliquots were brought to a rapid boil and titrated hot with 0.1 M NaOH using phenolphthalein as indicator.

*Determination and identification of esters.* At the start of this work the total ester content was determined by the petroleum ether extraction and saponification method described by Amerine (1944). Subsequently the Hestrin (1949) direct colorimetric method was used. Glucose and pyruvate in concentrations from 0.2 to 1 per cent interfere with this colorimetric method, and it was necessary to distill the ester from solutions containing these compounds.

The esters to be identified were obtained after 12 to 16 days' growth of *H. anomala* and *P. kluyveri* in 2 L of medium 2, containing 100 g of glucose. The yeast cells were filtered off, and the media were distilled under vacuum in an all glass still to remove the esters. The distillate obtained from the *H. anomala* culture medium, containing 8.7 mM ester per 100 ml, was concentrated by freezing and purified by extraction (three times) with alkaline water (Porter and Stewart, 1943) and treatment with saturated calcium chloride. The purified ester then was dried with anhydrous magnesium sulfate (Robertson, 1948). The physical and chemical properties of the purified ester from *H. anomala* given in tables 1 and 2 show the ester to be ethyl acetate. The acid portion of the ester also was shown to be acetic acid from the $R_f$ value obtained by a modification of the paper chromatography method of Fink and Fink (1949).[2]

Insufficient amounts of the ester produced by *P. kluyveri* were recovered for complete identification. The acid portion of the ester was acetic acid, but the alcohol portion was an alcohol other than ethanol.

TABLE 1

*Physical properties of the purified ester from Hansenula anomala*

| | PURIFIED ESTER | MALLINCKRODT ETHYL ACETATE | LITERATURE VALUES FOR ETHYL ACETATE |
|---|---|---|---|
| Boiling Point* | 75–79° | 74–77° | 77° |
| Refractive index $n_D^{20}$ | 1.3721† | 1.3722† | 1.37012‡ |
| Density $d_4^{25}$ | 0.8906 | — | 0.8943‡ |

* Micro-boiling point determination—as described in McElvain (1949).

† Bausch and Lomb—Abbe refractometer—Tungsten filament lamp 120 v 100 watts as light source.

‡ $n_D^{25}$ and $d_4^{25}$ from Griswold, Chu, and Winsauer (1949).

TABLE 2

*Chemical data on the ester from Hansenula anomala*

| MILLIEQUIVALENTS OF THE ISOLATED ESTER TAKEN | mM OF ALCOHOL RECOVERED* | mM OF VOLATILE ACID RECOVERED† | EQUIVALENT WEIGHT OF ESTER‡ |
|---|---|---|---|
| 1.28 | 1.35 | 1.08 | 92.7 |

* Calculated as ethanol.

† Calculated as acetic acid.

‡ Average of six determinations—literature value = 88.

*Volatile acid (acetic acid).* The ester present was evaporated off on the steam bath under slightly alkaline conditions, and the volatile acids present were separated and identified by the method of Duclaux as modified by Van der Lek (1930). The volatile acid in every instance was acetic acid only.

*Ethanol.* The method used was a modification of the acid dichromate method of Semichon and

[2] We wish to thank Dr. William Stepka for suggesting the use of a mixture of 74 ml butyl alcohol, 19 ml glacial acetic acid, and 50 ml water as solvent for the hydroxamic acids.

Flanzy (1929) and is similar to that used by Peel (1951). When more than 0.2 mM per 100 ml of ester was present, the ester was saponified and the ethanol present in the ester was subtracted from the total ethanol to obtain the free ethanol in the sample.

*Glucose.* Glucose was determined by the method of Somogyi (1945).

## RESULTS

(1) *Rate of aerobic and anaerobic ester production in glucose yeast autolysate (medium 2).* The anaerobic experiment was carried out under an atmosphere of $N_2$ in a 3 liter mercury sealed Erlenmeyer flask. The data for the experiment are given in table 3. It is seen readily that under aerobic conditions forty-one per cent of the ester was produced after all the glucose had disappeared. The ester is not produced directly from glucose by *H. anomala* but from ethanol. The majority of ester was produced after the acid reaction had dropped to pH 3 or below. The yield of ester after 12 days was 0.92 g from 5.2 g of glucose or 24 per cent on the basis of carbon utilized. The volatile acid increased gradually over a period of 12 days and then rose rapidly from 0.67 mM per 100 to 0.92 mM per 100. This rise may be due to oxidation of ethanol directly but is more likely due to hydrolysis of ester.

TABLE 3

*Rate of ester production by Hansenula anomala in glucose yeast autolysate (medium 2)*

| DAYS | GLUCOSE, G/100 ML | pH | ACETIC ACID, MM/100 | TOTAL ACID, MM/100 | ETHYL ACETATE,* MM/100 ML |
|---|---|---|---|---|---|
| *Aerobic condition* | | | | | |
| 0 | 5.2 | 4.8 | — | 1.56 | 0.002 |
| 1 | 4.85 | 4.1 | 0.13 | 1.75 | 0.09 |
| 3 | 2.98 | 3.4 | — | 2.30 | 0.88 |
| 4 | 2.18 | 3.3 | 0.30 | 2.33 | 1.30 |
| 6 | 0.32 | 3.1 | 0.49 | 2.80 | 4.40 |
| 8 | 0.017 | 3.1 | 0.62 | 2.90 | 6.80 |
| 12 | 0.024 | 2.4 | 0.67 | 3.25 | 10.50 |
| 14 | — | 2.5 | 0.92 | 2.64 | — |
| *Anaerobic condition* | | | | | |
| 0 | 5.2 | 4.8 | — | 1.56 | 0.002 |
| 3 | 4.72 | 4.1 | 0.37 | 1.46 | 0.005 |
| 13 | 3.60 | 3.7 | 0.25 | 1.62 | 0.01 |
| 24 | 2.45 | 3.7 | 0.27 | 1.75 | 0.003 |
| 35 | 0.55 | 3.5 | 0.42 | 1.97 | 0.035 |
| 39 | 0.29 | 3.6 | — | — | 0.05 |

* By the method of Amerine (1944).

No ester was produced by growing cells in the absence of oxygen. The amount of ethanol formed at the completion of the experiment was 1.94 g per 100 or a yield of 48 per cent on the basis of the carbon utilized. The amount of acetic acid formed anaerobically was small; some of it probably was introduced with the inoculum, and the remainder may have resulted due to an incomplete removal of oxygen from the fermentation flask. No fixed acid was formed anaerobically although aerobically about 1 mM per 100 ml was found. Aerobically the *H. anomala* film utilized the glucose completely in one-fifth the time necessary for anaerobic utilization.

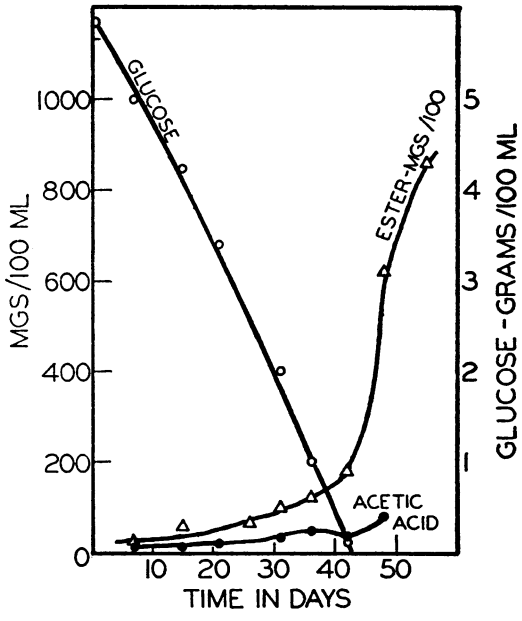


*Figure 1.* Rate of ester production by *Hansenula anomala* in a glucose, ammonium salt medium without growth factors. Ester and acetic acid in mg per 100 ml.

(2) *Ester production in a glucose synthetic medium (1a) without growth factors.* In this medium, with glucose as carbon source and ammonium salt as sole nitrogen source, 81 per cent of the ester was produced after the glucose had been utilized completely, as shown in figure 1. With regard to change in pH, volatile acid, total acid, etc., the results are similar to those described for these factors in the previous experiment. Al-

though this medium was a poor one for *H. anomala*, six weeks being required for complete utilization of glucose as contrasted with 8 days for medium 2, the yield of ester, however, remained unaffected. The ester yield here was 20 per cent (on the basis of carbon content) as compared with 24 per cent for growth in medium 2. Thus it appears that no specific nitrogen source or growth factor is especially beneficial to ester production, or what is more likely, *H. anomala* can synthesize all the enzyme complexes necessary for ester production from glucose, ammonium ion, and the usual essential inorganic elements. As a result of the poor growth in medium 1a, however, the time necessary for maximum ester production was prolonged. The effect on ester production of various growth media for *H. anomala* and *P. kluyveri* is shown in figure 2.

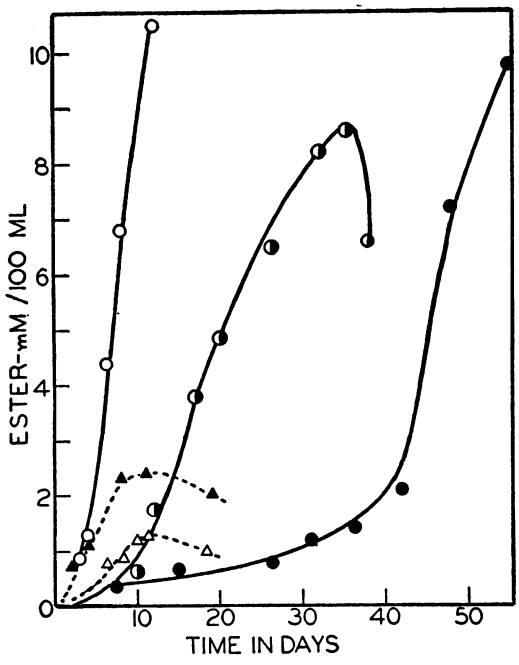


*Figure 2.* The effect of nitrogen source and growth factors on the yield of ester produced by *Hansenula anomala* and *Pichia kluyveri*.

Open circles = *Hansenula anomala* in 5 per cent glucose yeast autolysate medium.

Half-closed circles = *Hansenula anomala* in 1.8 per cent ethanol ammonium salt medium with growth factors.

Closed circles = *Hansenula anomala* in 5 per cent glucose ammonium salt medium.

Open triangles = *Pichia kluyveri* in 5 per cent glucose yeast autolysate medium.

Closed triangles = *Pichia kluyveri* in Wickerham's nitrogen base medium with 5 per cent glucose.

(3) *Ethanol as an "intermediate" in ethyl acetate production from glucose.* Yamada (1927) and Gordon (1950) postulate ethanol as the source of ethyl acetate but present no experimental evidence for their views. Figure 2 in Gray's paper (1949) shows that approximately 40 per cent of the ester in his experiment was formed after all the glucose had been utilized, but this fact is not mentioned in his discussion. Peel (1951) was the first to report the production of ester from ethanol.

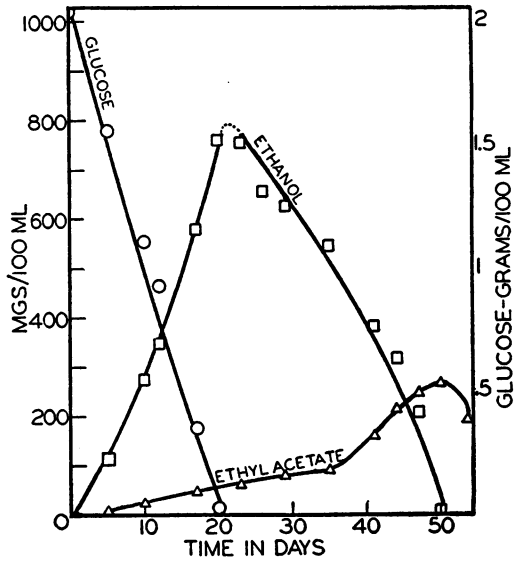


*Figure 3.* Illustrating the direct production of ethyl acetate from ethanol accumulated in the fermentation of glucose by *Hansenula anomala* in medium 1. Ethanol and ethyl acetate in mg per 100 ml.

Figure 3 illustrates clearly the direct formation of ethyl acetate from the ethanol produced in the utilization of glucose in medium 1. At first there was a rapid increase in ethanol production up to the 20th day; all of the glucose was utilized during this interval. Twenty per cent of the ethyl acetate was formed slowly during this time. After this period, there were a relatively rapid utilization of the ethanol produced and a corresponding fivefold increase in the rate of ethyl acetate pro-

duction. Since ethanol accumulated in the medium during glucose utilization and there is a three day lag before the ethanol is actively respired, it is highly probable that some of the enzymes involved in ethanol utilization are adaptive in nature.

The maximum yield of ethanol including that transformed into ethyl acetate was 50.6 per cent. The yield of ethyl acetate was 17.8 per cent. The yeast film apparently utilized some of the free acetic acid since its value here never rose above 0.48 mM per 100 ml. The total acid and pH changes were similar to those in the previous experiments.

(4) *The production of ethyl acetate with ethanol as sole carbon source.* The changes occurring in medium 1, with 1.8 per cent ethyl alcohol as sole carbon source, typical of the data obtained for the direct production of ethyl acetate from ethanol, are shown in figure 4. The curve of interest in figure 4 is the acetic acid curve. Up to the 17th day there was a slow increase to 75 mg per 100 ml of acetic acid. For the next nine days there was a slow utilization of acetic acid and finally a sharp increase coincidental with the rapid hydrolysis of ethyl acetate. The largest amount of free acetic acid obtained was 348 mg per 100 ml. The major portion of this acid appears to arise directly from the hydrolysis of the ester as can be seen readily from comparing the ethanol and acetic acid curves in figure 4. Figure 4 shows no accumulation of ethanol but instead an increase in acetic acid during ester hydrolysis. The leveling off of the acetic acid curve after the 44th day is probably caused either by utilization of acetate or death of the cells in the yeast film.

Since 5 mM per 100 ml of acetic acid at pH 2.4 to 2.6 (pH for the last 24 days of the experiment) is usually toxic to *H. anomala* (as determined from cell suspension experiments), the cells in the film were examined periodically and a vital stain was made using 1:10,000 methylene blue at pH 4.6. At the conclusion of both this and the glucose experiment (figure 3), 90 per cent of the cells grown on glucose was found to be viable by vital stain, the rest being granular and distorted. The cells grown in the ethanol medium were 100 per cent dead after 35 days. A check by viable count on wort agar plates, however, showed 16 per cent of these ethanol grown cells to be alive. Except for these few resistant cells, it can be said that about 5 mM per 100 ml of acetic acid at pH 2.4 is toxic to *H. anomala*.

The fixed acid produced from ethanol was 1.6 mM per 100 ml. The maximum yield of ester was 44.5 per cent on the basis of carbon utilized.

(5) *Effect of excess aeration on ester production.* This experiment was conducted with 1,000 ml of medium 1 using the shake culture technique identical with that used in obtaining active cell

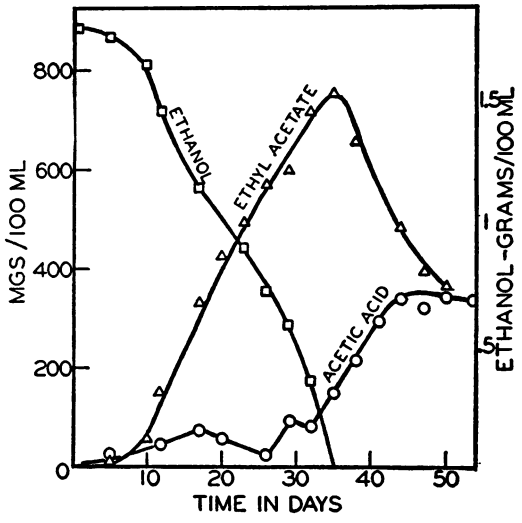


*Figure 4.* The direct production of ethyl acetate from ethanol by *Hansenula anomala* and the increase in acetic acid concentration as the ester is utilized. Ethyl acetate and acetic acid in mg per 100 ml.

suspensions. The relatively rapid glucose utilization in this experiment (figure 5) demonstrates that availability of oxygen, as well as lack of certain growth factors, may have been one of the limiting factors with regard to glucose utilization in the previous experiments with medium 1.

The total ethanol produced from 2 per cent glucose was only 13 per cent of that formed in the standing culture experiment (figure 3). The production of ethyl acetate was also lower than with the standing culture experiments, being 57 per cent of that formed in the standing culture experiment with medium 1. The lower yield of ester is not surprising since ethanol production also is lower under the above experimental conditions. One liter of a water solution containing 3.78 mM per 100 of ethanol and 1.60 mM per 100 ethyl acetate, respectively, was shaken for 12 hours. After 12 hours the results showed that

2 per cent of the ester had been lost by evaporation and 9.3 per cent of the ethanol. This loss is not large enough to account entirely for the rapid decreases in these compounds within the 4 hour sampling periods of the actual experiment.

There was a relatively large amount of free acetic acid (4.28 mM/100) produced in 50 hours. From the ethanol and ethyl acetate curves of figure 5, it would appear that much of the acetate arose from incomplete oxidation of ethanol. The amount of fixed acid produced here was 1.72 mM per 100 and the pH dropped to a low of 2.2.

The ester odor emitted by these cultures reminded one of the heady odor of gardenias, but odor alone is not sufficient for distinguishing esters. The typical sharp odor of ethyl acetate was easy to identify when *H. anomala* was grown in an ethanol medium 1, but this odor was masked upon growth in other media. In medium 1 with glucose, the odor was flower-like, whereas in medium 2 containing yeast autolysate, the apple-like odor described by earlier investigators (Hansen, 1891) was noted. The type of odor emitted during growth of the organism appears

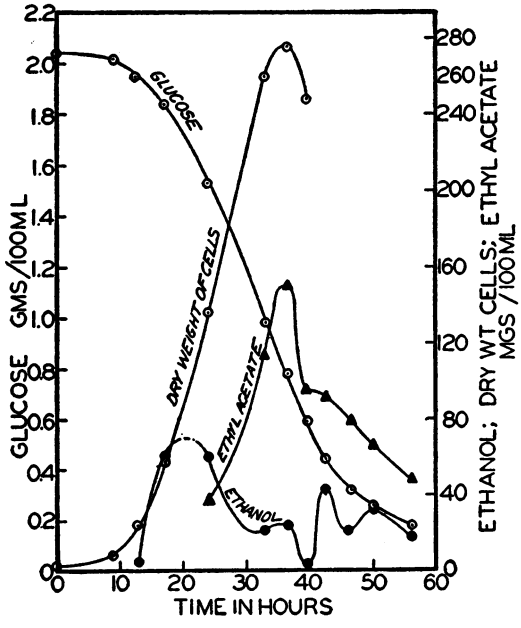


*Figure 5.* The effect of excess aeration on ester production by *Hansenula anomala.*

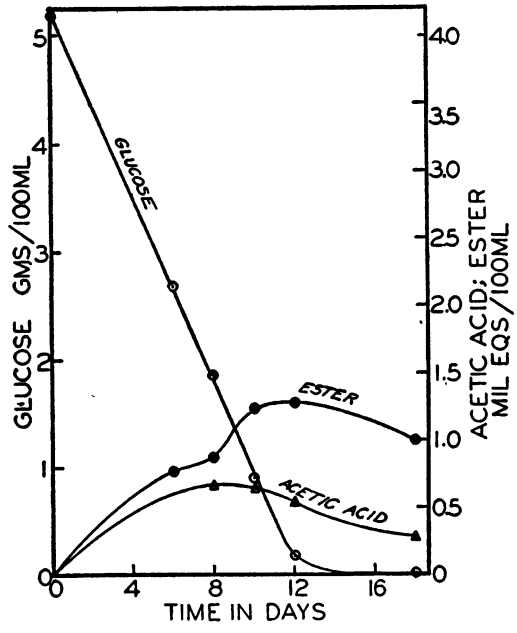


*Figure 6.* Ester production by *Pichia kluyveri* in a 5 per cent glucose yeast autolysate medium.

The results of this experiment combined with those for the standing cultures show, as was pointed out by Gray (1949), that there is a critical $O_2$ tension at which maximal production of ester can occur. The other very important factor in high yields of ester, i.e., pH, will be discussed in a later publication dealing with cell suspension experiments.

(6) *Ester production by Pichia kluyveri in media 2 and 3.* The results obtained for standing surface cultures of *P. kluyveri* in media 2 and 3 are shown in figures 6 and 7. These show that the ester formed by *P. kluyveri* was produced directly from glucose or from some product formed early in glucose utilization.

to depend upon both the carbon and nitrogen sources present in the medium.

Although the glucose was utilized at a slightly more rapid rate in medium 2, twice as much ester was produced from the 5 per cent glucose in medium 3 (figure 7), which may contain one or more factors beneficial to ester production. The maximum yield of ester produced by *P. kluyveri* was approximately one-fifth of that formed by *H. anomala.* Absolute values for ester cannot be determined since the identity of the alcohol portion of the ester remains unknown. The ester produced by *P. kluyveri* does not appear to be ethyl acetate (see above). This organism can produce the ester with ethanol as sole carbon

source in medium 2, but the ester formed has not been identified.

In both media 2 and 3, the acetic acid decreased simultaneously with ester utilization (figures 6 and 7). Apparently both ester and acetic acid may be utilized readily by *P. kluyveri* when all the glucose has disappeared. Twice as much acetic acid was formed in Wickerham's medium 3. Very little total acid was formed in either medium, and in medium 2 some of this acid was utilized during the last six days. The pH of the medium

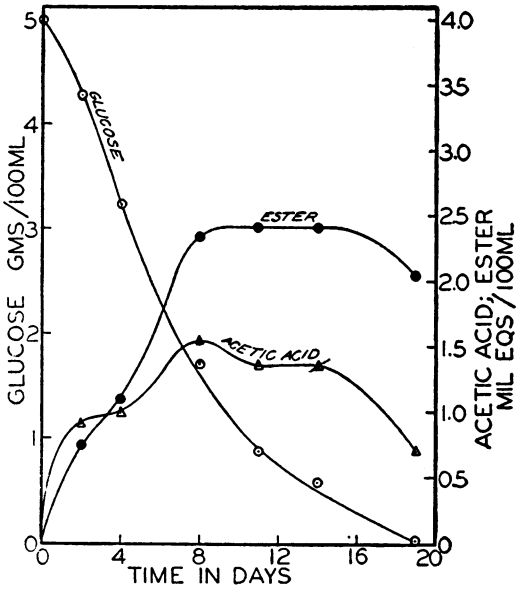


*Figure 7.* Ester production by *Pichia kluyveri* in 5 per cent glucose Wickerham's nitrogen base medium.

dropped rapidly to 2.5 but in medium 2 there was a slight rise to pH 3 during the last six days.

## DISCUSSION

The experiments described above with growing cultures of *H. anomala* show that ethyl acetate is a direct product of ethanol oxidation. Apparently any carbon source which *H. anomala* can utilize with a resultant production of ethanol will result also in ethyl acetate production, provided sufficient ethanol is formed and the other requirements ($O_2$ tension, low pH) for ester production are met.

The fact that oxygen tension has an important influence on ester production was confirmed. A literature survey by Tabachnick (1950) of pure cultures of ester producing organisms showed all such organisms to be predominantly aerobic. In a survey of various ester producing yeasts and molds it was found that the acid portion of the isolated esters was in all instances acetic acid. Strict anaerobic conditions result in poor growth with *H. anomala* and no ester production, while an excess of air may result in either low ethanol production or perhaps a rapid oxidation of both ethanol and any ester which may be formed. With the proper amount of aeration, *H. anomala* can give yields of ester as high as 48 per cent of theoretical with ethanol as substrate. Some of the energy made available by ethanol oxidation probably is used in forming the ester linkage.

The aerobic production of ethanol observed with this organism is not an unusual phenomenon. The aerobic production of ethanol from glucose by various molds was noted by Foster (1949) and in yeast by Custers (1940) under conditions of aeration even more efficient than those used here. With *H. anomala* approximately one-third as much ethanol was produced per gram of glucose consumed aerobically. In the standing culture experiments semiaerobic conditions prevailed, for some of the yeast film settled to the bottom of the Fernbach flasks and gas bubbles were seen to rise from the bottom of the flask. The cells in the surface film, however, were more numerous and probably carried out the major portion of both ethanol and ethyl acetate production from glucose, for, as has been observed, a very low oxygen tension results in very slow glucose utilization and little ester formation.

Peel (1951) calculated that the concentration of ethyl acetate in equilibrium with the observed concentrations of ethanol and acetic acid present in a culture medium would be $1.5 \times 10^{-5}$ M instead of $46 \times 10^{-3}$ M actually found. Our values for ethyl acetate production similarly are one thousand times greater than calculated equilibrium values, but since there is no evidence of the existence of a true equilibrium between the ethanol formed from glucose or initially added and the ethyl acetate and acetic acid produced, this is not sufficient evidence against a mechanism involving esterase catalyzed hydrolysis. Other evidence against a mechanism involving a reversal by an esterase consists of: (1) the direct production of large amounts of ethyl acetate with ethanol as sole carbon source and (2) the

need for some unspecified amount of oxygen for maximum yields of ester. Both Gray (1949) and Davies *et al.* (1951) found acetaldehyde present in small amounts in the spent glucose medium upon which *H. anomala* had grown. This is not sufficient evidence for implicating acetaldehyde as an intermediate in ethyl acetate formation. Peel (1951) reported that acetaldehyde is not directly converted to ester but in low concentrations does stimulate ester production from ethanol. Contrary to previous reports, the amounts of growth factors and the nitrogen source do not affect significantly the yields of ethyl acetate produced by *H. anomala*. In a complete medium, however, the substrate is consumed more rapidly, and as a consequence the maximum yield of ester may appear two weeks sooner than in a less favorable medium.

Although ester production appears to occur over a fairly wide pH range (4.4 to 2.2), the highest yields of ester are obtained below pH 3. The importance of low pH in the production of high yields of ester was not made clear until work with cell suspensions had been conducted (*unpublished data*). At this low pH, relatively low concentrations of acetic acid are toxic to this organism, and as suggested by Gordon (1950) for *Endoconidiophora moniliformis*, the formation of ethyl acetate may protect the organism from the toxic effects of acetic acid. In this instance ethanol would be acting as the detoxifying agent rather than acetate, which is known to have a detoxifying function in animal metabolism.

The rapid utilization of the ethyl acetate produced by *H. anomala* has been observed by other workers (Kayser and Demalon, 1909; Will, 1913; Gray, 1949; Peel, 1951). The fact that acetic acid accumulates during ester utilization implies that the ethanol portion of the hydrolyzed ester is preferred by the yeast. Very little experimental evidence (Will, 1913) is available on the mechanism for ethyl acetate utilization by *H. anomala*.

The fixed acid formed by *H. anomala* in the media used averaged 1 mM per 100 ml. A fractionation of the acids present (Hyams, 1950) by paper chromatography resulted in the identification of spots for fumaric and citric acids and a spot which may be succinic or lactic acid. Under anaerobic conditions no fixed acid was formed. Aerobic $C^{14}O_2$ fixation experiments[3] with cellular suspensions of this organism resulted in radioactivity appearing in spots identified as succinic, malic, citric, and fumaric acids, these acids of course being extracted from within the cells. The above data appear to indicate that a Krebs-like respiratory mechanism may exist in *H. anomala* for the oxidation of ethanol as well as other substrates.

The experiments with growing cultures of *P. kluyveri* show that the results obtained with *H. anomala* are unique for this organism and are not applicable to other ester forming yeasts. The ester formed by *P. kluyveri* has not been identified. The amount of ester formed was small (1/5 that produced by *H. anomala*). The significant increase in ester yield when the cells were grown in Wickerham's synthetic medium as compared to the lower yield with equally good growth in a yeast autolysate medium implies that *P. kluyveri* may be a good organism to use in investigating the need for cofactors which may be involved in ester bond synthesis.

[3] We wish to thank Dr. V. H. Lynch for performing these experiments.

ACKNOWLEDGMENTS

We wish to express our thanks to Professor H. A. Barker and M. Doudoroff for helpful suggestions made throughout the course of this work.

SUMMARY

With growing cultures of *Hansenula anomala* and glucose as carbon source, it was shown that ethyl acetate, the ester produced by this organism, was formed as a result of the aerobic utilization of ethanol accumulated in the fermentation of glucose. The ester, in turn, was utilized rapidly under aerobic conditions.

The same high yields of ester were obtained regardless of the type of nitrogen source added. However, in a nutritionally complex medium containing glucose, which favored a high growth rate, the maximum yield of ester was obtained within 8 days as contrasted to 50 days in a medium in which ammonium salts were used as sole source of nitrogen.

The high yields of ester obtained leave no doubt that the formation of ester is an energy consuming reaction and not the reversal of a simple esterase hydrolysis.

Since free acetic acid is toxic to *H. anomala* at the acid pH (2.4 to 3.0) for optimal ester yields, it would appear that the formation of ethyl acetate has survival value for this or-

ganism by preventing the accumulation of toxic amounts of acetic acid in the medium.

Experiments with growing cultures of *Pichia kluyveri* indicate that other microorganisms may produce esters other than ethyl acetate directly from glucose or some early product of glucose.

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