A STUDY OF THE MECHANISM OF
SYNTHESIS OF ETHYL ACETATE BY
HANSENULA ANOMALA

by

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Introduction

There are three basic reasons or justifications for the study of the mechanism of synthesis of a compound by a micro-organism. The first and most easily justified is that the information gained by such an investigation may in some way be applied to a similar system in mammalian physiology. This information would enable one to more clearly understand the normal functions and relationships of the cells comprising the bodies of higher animals. An understanding of the normal functions and metabolic relationships would afford one a better insight into the changes brought about by a disease process.

The second reason would be from a purely economic standpoint. The information obtained concerning the mechanism and the factors influencing the synthesis of a compound by a living microorganism may prove of value in the commercial production of this compound. If so, this would justify such an investigation.

The third would be on the basis of academic interest. Knowledge concerning metabolic activities of microorganisms would increase understanding of the environment in which man lives.

A study of the mechanism of synthesis of ethyl acetate by Hansenula anomala could satisfy the above three criteria. The synthesis of acetylcholine by mammalian cells and ethyl acetate by a yeast may prove to have common similarities. Another possibility exists between the study of this common saprophyte and human disease. It is a common observation that there is a fruity odor associated with a culture of Candida albicans, a

human parasite. What association, if any, there is between the production of volatile esters and acids by this pathogen and its ability to produce disease in animals is a question to be answered.

An understanding of the mechanism of formation of ethyl acetate by H. anomala may make the production of ethyl acetate by this yeast economically feasible.

Relatively little is known of the metabolic activities of a great many of the common yeasts found in nature. Increased study of these organisms appears to be needed.

LITERATURE REVIEW

Rommier (1890) made one of the first recorded observations of esters being produced during fermentation processes. He reported that the fatty acids and alcohols produced during fermentation combined frequently while in the nascent state to form esters. Kayser (1890) reported that a top yeast, Saccharomyces mali, imparted a pleasing bouquet to cider. The same author (1891) reported the isolation from a pineapple of an unnamed Mycoderma which produced an ester-like odor. Hansen (1894) in his original description of "Saccharomyces anomalus" commented that during the course of fermentations conducted with this yeast a strong odor of fruit essence was emitted. This yeast has been placed in the genus Hansenula and the preferred name is Hansenula anomala (Hansen) Sydow (Bedford, 1942).

Other species of Hansenula apparently produce esters. Klöcker (1903) mentioned the presence of an ester in fermentations with Hansenula saturnus (Klöcker) Sydow (Saccharomyces satunus Klöcker). Klöcker suggested that the ester produced might have been ethyl acetate but offered no proof. He also stated that levulose-containing medium fermented by H. saturnus had a strong odor of the "ether de poires", indicating the presence of amyl acetate. Zikes (1906) reported the formation of esters in connection with fermentations by H. wichmanni (Zikes) Sydow (Willia wichmanni Zikes).

Seifert (1897) stated that Hansenula anomala synthesized ethyl acetate in beer wort. He offered no proof for this statement.

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Steuber (1900), who studied several varieties of the species H. anomala, found that some produced esters while others did not. He, like Seifert, gave no evidence indicating the identity of the esters nor did he give any data concerning the quantity in which they were produced. Guilliermond (1912) reported that members of the genus Hansenula produced esters and that H. anomala synthesized ethyl acetate. This statement was made without substantiation.

Zikes (1906) stated that according to a communication of Nielsen, Hansen's strain of H. anomala produced ethyl acetate to a concentration of 0.9 per cent. Bedford (1942) reported that the ester had been quantitatively determined in H. anomala fermentations but presented no data.

Gray (1949) made a study of the metabolism of Hansenula anomala (Hansen) Sydow. He concerned himself with the optimal initial pH of the fermentation medium for cell multiplication, for most rapid glucose utilization and for maximum ester production. He also studied and characterized the ester and the other volatile neutral metabolic products, the effect of aeration on ester synthesis, the effect of various carbon compounds on ester formation, and the effect of the concentration of yeast extract on total ester production.

In a medium consisting of 0.7 per cent Difco yeast extract, 0.5 per cent KH_2PO_4, and 5 per cent glucose, he found that the optimal initial pH for cell production, for most rapid glucose utilization and for maximum ester synthesis, was between 5.0 and

6.0. Ester determinations were made by distilling the fermentation medium, adjusting the distillate until it was just pink to phenolphthalain indicator, refuxing with 0.1 N NaOH for one hour and titrating the cooled refluxate with 0.1 N HCL. Ester content (as ethyl acetate) was calculated from the amount of NaOH solution used in the neutralization of the acid freed during saponification of the ester.

By varying the concentration of the yeast extract between 0.1 and 1.5 per cent, Gray found the concentration for best ester production was 0.7 per cent.

The ester produced by H. anomala was identified by the preparation and characterization of ethyl p-nitrobenzoate and p-nitrobenzyl acetate from the acid and alcohol found in the saponified distillate. Since the derivatives of both ethyl alcohol and acetic acid were prepared from the saponified ester obtained by fractional distillation of the culture filtrate, there was little doubt that the ester was ethyl acetate.

An attempt to determine the effect of aeration on ester production by H. anomala was made by Gray (1949). Five 100 ml. portions of media were pipetted into Erlenmeyer flasks of 125, 250, 500, 1000 and 2000 ml. capacity. The area/volume ratios of the flasks were calculated for each flask. These ratios gave an index to the degree of aeration of each flask. Each flask was inoculated with 0.1 ml. of liquid cell suspension and incubated for 5 days. At the end of this period, ester determinations were made. It was concluded that increased aeration by increasing

the surface/volume ratio resulted in a decided decrease in ester production. Exactly the opposite effect was found by another investigator (Larsen, 1950) employing aeration and agitation.

An attempt was made by Gray (1949) to increase yields of ester by the substitution of other carbon compounds for glucose. These were added in the same concentrations (5 per cent) to the fermentation medium. These compounds were soluble starch, various monosaccharides (both hexoses and pentoses), sodium acetate, ethyl alcohol, sucrose and ammonium lactate. The initial pH of these various media ranged between 5.6 and 8.8. He found that highest ester yields were obtained in media containing d-mannose, d-glucose, sucrose and ammonium lactate. It is interesting that the highest yield of ester was attained in the medium containing ammonium lactate. From 25 ml. of the ammonium lactate culture filtrate 221.76 mg. of ester were found as compared to 189.38 mg. from the glucose culture filtrate. Gray suggested that the better growth and ester production may have been the result of additional nitrogen supplied by the ammonium lactate. However, he suggested that this effect should be investigated more thoroughly since it might throw some light on the mode of synthesis of ethyl acetate by Hansenula anomala.

Larsen (1950) made an extensive study of ester forming yeasts and compared them to non-ethyl acetate forming yeasts. In his study he used three strains of H. anomala, two strains of Saccharomyces cerevisiae, and one strain each of Kloeckera apiculata, S. aromaticus, Torula sphericus, and Candida albicans.

In a completely synthetic medium composed of trace and macro
amounts of salts, vitamins, and glucose as a carbon source,
Larsen found that the ester producing yeasts could utilize either
0.08 per cent KNO_3, 0.1 per cent $(NH_4)_2SO_4$, 0.05 per cent urea,
0.1 per cent asparagine or 0.13 per cent peptone as a sole source
of nitrogen. The non-ethyl acetate producing yeasts differed
only in that they failed to grow when KNO_3 was the only nitrogen-
containing compound present.

Carbon assimilation tests were conducted in the same manner
as the nitrogen assimilation experiments. The nitrogen source
employed was $(NH_4)_2SO_4$ at a concentration of 0.5 per cent. The
carbon sources were present in a concentration of 1.0 per cent.
The 5 ethyl acetate producing yeasts were similar in their abil-
ities to utilize a wide variety of carbon compounds for growth.
The non-ethyl acetate producing yeasts differed considerably in
their abilities to utilize the same compounds. Aeration and
agitation by shaking did not permit the yeasts to assimilate any
compound which was not assimilated when grown in undisturbed
test tubes.

Larsen was unable to demonstrate a vitamin deficiency in
any of the ethyl acetate forming yeasts as far as biotin, panto-
thenic acid, inositol, niacin, p-amino benzoic acid, pyridoxine,
thiamine or riboflavin were concerned. At least one deficiency
was demonstrated for each non-ethyl acetate forming yeast.

It was demonstrated that agitation and aeration influenced
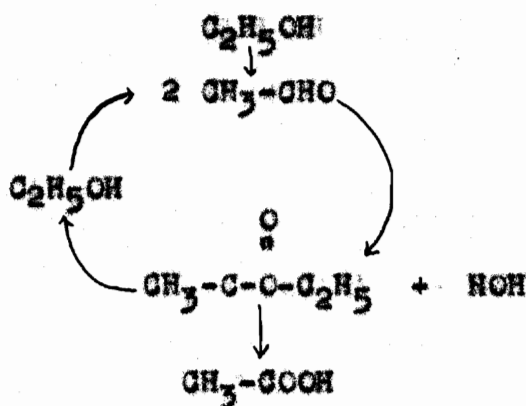the ratio of alcohol and ester formed, as well as growth and

the amounts of sugar utilized by H. anomala. For a non-agitated deep culture a thirteen day incubation period was required for complete utilization of sugar in a 0.6 per cent glucose medium at which time 6.64 per cent ethyl acetate (calculated from glucose) was produced. Agitation with a plentiful oxygen supply resulted in more rapid sugar utilization and at the end of 58 hours 19.0 per cent ethyl acetate was recovered. This finding contradicts that of another worker (Gray, 1949) who stated that aeration of a culture of H. anomala resulted in less ester production.

Davies et al. (1951) has substantiated the findings of Gray (1949) that ethyl acetate, ethyl alcohol, acetic acid and acetaldehyde are products of the metabolism of glucose by H. anomala. Their strain of H. anomala produced about 25 m mol of ethyl acetate per 100 m mol of glucose utilized under aerobic conditions. They further verified the findings of Larsen (1950) concerning the aerobic formation of ethyl acetate by H. anomala.

Peel (1951) found that under aerobic conditions washed suspensions of H. anomala formed ethyl acetate from ethyl alcohol alone or in the presence of acetate. This substantiated Larsen's report (1950) that added acetate does not increase the yield of ester. Peel also found that the ester formed disappeared after exhaustion of the ethyl alcohol.

The exact mechanism of formation of ethyl acetate by H. anomala is not known. Wolf and Wolf (1947) commenting on the formation of ethyl acetate by Penicillium digitatum, suggested that it originates from acetaldehyde by a Cannizzaro reaction.

This, however, would not be a Cannizzaro condensation reaction but a Tischtschenko reaction. A Cannizzaro reaction is the condensation of two molecules of an aldehyde (with an α-hydrogen) and similtaneous saponification with sodium hydroxide, while a Tischtschenko reaction is the condensation of two molecules of an aldehyde (with or without an α-hydrogen) to form one molecule of an ester (Cheronis, 1943). Gray (1949) postulated that ethyl acetate formation by H. anomala may occur in a similar manner. Larsen (1950) conjectured that it may be mediated through a Tischtschenko-like reaction. He proposed a scheme in which the ester would represent an intermediate in the oxidation of ethyl alcohol to acetic acid. The proposed scheme is as follows:

$$C_2H_5OH$$
$$\downarrow$$
$$2 \ CH_3-CHO$$

$$C_2H_5OH$$

$$\overset{O}{\overset{\|}{CH_3-C-O-C_2H_5}} \ + \ HOH$$
$$\downarrow$$
$$CH_3-COOH$$

Each complete cycle would result in the oxidation of one molecule of ethyl alcohol to acetic acid. Larsen also suggested that formation of ethyl acetate might be by esterification of ethyl alcohol by acetic acid which might prevent the accumulation of a high concentration of acetic acid. Peel (1951) stated that

his results showed that theamounts of ester produced by washed suspensions of H. anomala were too large to be accounted for by the reversal of a simple esterase reaction. He maintained that his data suggested a more exergonic mechanism of ester formation. This was confirmed, he said, by the fact that under anaerobic conditions no ester was formed from ethyl alcohol plus acetate or from acetaldehyde, showing that oxidation was necessary for the synthesis of ester. He suggested that it was possible that oxidation of ethyl alcohol may have lead to the formation of a labile acetyl derivative which then reacted with further ethyl alcohol to produce the ester.

It may be concluded that from the information available in the literature the exact mechanism of ethyl acetate synthesis by H. anomala and its role in the metabolism of the organism is as yet unknown.

EXPERIMENTAL METHODS AND MATERIALS

Cultures

A subculture of the organism used in this study was obtained from Dr. Don H. Larsen. He gave it the designation *Hansenula anomala* 7. This terminology will be used in this investigation. Stock cultures were maintained on a medium consisting of 0.5 per cent peptone, 0.2 per cent yeast extract, 1.0 per cent glucose and 2.0 per cent agar. The pH was adjusted to approximately 5.5 prior to sterilization. All media were sterilized in the auto-clave at 15 pounds pressure for 15 minutes. Cultures were incubated at room temperature for 48 hours and stored in the refrigerator. Transfers to fresh media were made monthly.

Chemicals

The chemicals used in this study were, when possible, C.P. grade or better. Reagent grade chemicals were used in the preparation of reagents for analytical procedures. The isopropyl, n-propyl, isobutyl, n-butyl and t-butyl alcohols, and the propionaldehyde obtained from the Department of Chemistry, University of Utah, were of questionable purity. The C^{14}-labelled sodium acetate, kindly supplied by Dr. Leo T. Samuels, Department of Biochemistry, University of Utah, was originally obtained from Tracerlab Inc., Boston, Mass. This compound was labelled with carbon-14 in the methyl position and was stated to have a specific

activity of 1 millicurie per millimole. The adenosine triphos-
phate (ATP) used in this study was supplied through the courtesy
of Dr. Robert Recknegel of the Department of Pharmocology, Uni-
versity of Utah. It was in the form of the sodium salt. A
standard solution of this compound was prepared in M/15 buffer
at a pH of 7.0 and stored in the cold. The diphosphopyridine
nucleotide (DPN), supplied by Dr. Leo T. Samuels, was also in
the form of the disodium salt and was treated in the same manner
as the ATP.

Glassware Preparation

With the exception of volumetric equipment and flasks used
in the oxygen uptake experiments, all glassware was soaked in a
solution of commercial washing powder, rinsed in tap water, rinsed
in an acid bath and further rinsed in distilled water. Volumet-
ric glassware and Warburg flasks were cleaned in chromic acid
solution, repeatedly rinsed in distilled water and drained.

Preparation of Inoculum

Unless otherwise noted the inoculum for all media consisted
of cells harvested from 24 hour cultures on Sabouraud's maltose
agar slants. These slants were seeded from the stock agar cul-
tures. Ten ml. of sterile isotonic NaCL solution were added and
the cells scraped from the surface with a sterile inoculating
needle and suspended. Approximately 1 ml. of this suspension
per 100 ml. of culture media was used as an inoculum.

Incubation and Measurement of Growth

All cultures were grown at room temperature, 22° to 25° C., for varying lengths of time. The period of growth was variable and is described under the experimental results. Measurements of growth of cells were made by determining the per cent of packed cells in Wintrobe hematocrit tubes. These tubes were filled to the mark with the liquid culture and centrifuged at high speed for 60 minutes. After centrifugation the percentage of packed cells was read. While this technique was but a gross approximation, it was found that it gave a relative indication of the amount of growth in liquid cultures of the organism used in this study. Measurements of turbidity after growth were made by using a Klett-Summerson photoelectric colorimeter. A purple, number 42 filter was used.

Growth of H. anomala Cells

The medium used for obtaining cells of H. anomala 7 for enzyme isolation was composed of 0.5 per cent peptone, 0.1 per cent yeast extract, 0.1 per cent potassium acid phosphate and 1.0 per cent glucose. This solution was adjusted to pH 5.5 prior to sterilization. It was found that approximately 750 ml. of media could be placed into a 4 liter Erlenmeyer flask and shaken on a mechanical shaker without splashing up to the cotton stopper. Approximately 350 ml. of media could be shaken in a 2 liter flask without excessive splashing. Later in this study it was found that up to 5 liters of media could be shaken in a large Pyrex carboy with a capacity of 20 liters. Two of

these vessels could be placed on a mechanical shaker resulting
in excellent agitation and aeration. After the media was brought
to a pH of 5.5 the appropriate volumes were placed in flasks and
sterilized at 15 pounds pressure for 15 minutes. Upon cooling
the media the flasks were inoculated with a suspension of cells
washed from 24 hour stock agar slant cultures. After a period
of growth on a shaker of from 36 to 48 hours, the cells were
harvested by centrifugation. The further treatment of these
cells will be described under the experimental results.

pH Determinations

All pH determinations in this study were made with a Beck-
man model G glass electrode pH meter.

Ester Recovery; Determination of Ester

In the experiments involving the effect of various alcohols
and propionaldehyde on ester synthesis by H. anomala 7 the pro-
cedure used in an attempt to isolate esters was a modification
of that described by Shriner and Fuson (1940). The original
description was for the separation of acetic acid, n-butyl alcohol,
n-butyl acetate and sulfuric acid from the reaction mixture for
the preparation of n-butyl acetate. The modifications of this
method are described under the experimental results.

The quantitative determination of ethyl acetate was made
by a colorimetric procedure developed by Hestrin (1949). It is
based upon the density of color developed by the ferric-hydrox-
amic acid complex formed from esters with alkaline hydroxylamine.

The procedure was originally developed from the colorimetric determination of acetylcholine and related compounds. Hestrin (1949) reported the chromogenic activity of ethyl acetate per equivalent relative to acetylcholine to be 97.

The procedure consisted of mixing 2 ml. of a solution of equal volumes of 2 M hydroxylamine hydrochloride and 3.5 N sodium hydroxide with 1 ml. of the solution to be assayed. After at least one minute the pH was brought to 1.2 ± 0.2 with 1.0 ml. of concentrated hydrochloric acid diluted with 2 parts by volume of distilled water. Addition of 1 ml. of a 0.37 M solution of ferric chloride in 0.1 N hydrochloric acid resulted in color development. This color density was read in a Klett-Summerson photoelectric colorimeter with a green, number 54 filter. The micromoles of ethyl acetate per ml. of unknown samples were determined by reference to a standard curve prepared from known quantities of pure ethyl acetate. The effects of various buffers and hydrogen ion concentrations on the determination of ethyl acetate by this technique will be described in the section on experimental results. In assaying an enzyme preparation for activity the procedure for ester determination was carried out in centrifuge tubes. After the addition of the ferric chloride solution the mixture was centrifuged at high speed for a few minutes. This was necessary to remove precipitated protein and other particulate material which might interfere with the colorimetric assay procedure.

Radioactivity Determinations

Determination of radioactivity due to carbon-14 was made using a type A Geiger-Müller counter built by Cyclotron Specialties Co., Moraga, California. The tube used was a Mark I, model 3, manufactured by Radiation Counter Laboratories, Chicago, Illinois. It's mica window had a weight of 1.6 mgm per square centimeter. The methods of preparing the samples for counting are described in the section on experimental results.

Oxygen Uptake Methods

Oxygen uptake by resting cells of H. anomala 7 in the presence of various substrates and inhibitors was measured with a Warburg apparatus. The cells were harvested from 48 hour cultures on Sabouraud's-maltose agar slants and washed three times with distilled water. After washing with distilled water the organisms were suspended in a buffered salt solution described by Larsen (1950). The constituents of this solution are listed in Table I. The pH of this solution was adjusted to 4.5. After adjustment of the suspension to the proper turbidity it was aerated on a mechanical shaker for 30 minutes. The water bath of the Warburg apparatus was at a temperature of 30° C. and an oscillation rate of approximately 90 cycles per minute was used in all experiments. The kinds of substrates and inhibitors, their concentrations and other details are described in the experimental results.

Table I.

Constituents	Amount per liter
Trace salts	
H_3BO_3	50 ugm'
$CuSO_4 \cdot 5\ HOH$	40 ugm
KI	120 ugm
$FeCl_3 \cdot 6\ HOH$	250 ugm
$ZnSO_4 \cdot 7\ HOH$	200 ugm
$MnCl_2 \cdot 4\ HOH$	40 ugm
$Na_2MO_4 \cdot 2\ HOH$	25 ugm
$CoSO_4$	25 ugm
Macro salts	
KH_2PO_4	1.0 gm"
$MgSO_4$	0.5 gm
NaCl	0.1 gm
$CaCl_2 \cdot 2\ HOH$	0.1 gm

'ugm=microgram
"gm= gram

EXPERIMENTAL RESULTS

Influence of Buffer Composition and pH Upon the Colorimetric
Assay for Ethyl Acetate

Hestrin (1949) has reported that the ferric complex of
acethydroxamic acid showed a maximum absorption at between 520
and 540 mu. In this range of wave length ferric ion showed
little or no absorption. He found that the hydrolytic products
of acetylcholine did not alter the development of color by acetyl-
choline with alkaline hydroxylamine. The effect of certain iron-
binding anions on the reaction was studied by Hestrin (1949).
Under the conditions described in his procedure, the interfering
effect of a total of 50 uM of phosphate was not more than 3 per
cent in the test range of from 0.2 to 2.0 uM of acetylcholine in
5 ml. of the final mixture. Under the same conditions, 50 to
200 uM of sulfate or 100 uM of borate produced little or no
interference.

Studies of the influence of certain buffers on the colori-
metric estimation of ethyl acetate by the above method were made.
The buffer solutions studied were M/15 phosphate at pH 6.3 and
5.5, 0.004 N acetate buffer at pH 4.5 and a buffer solution of
M/20 Borax ($Na_2B_4O_7 \cdot 10$ HOH) plus a M/5 boric acid solution con-
taining 2.93 gm per liter of sodium chloride. The preparation
of this latter buffer solution was described by Clark (1928).
The pH of this solution was adjusted to 8.3. Standard solutions
of ethyl acetate were prepared in the above buffers and in

distilled water. A comparison of the curves obtained by plotting
Klett units against ester concentration is found in Figure 1.
While the slopes of the curves differ somewhat, it was assumed
that they were entirely within the experimental error of the
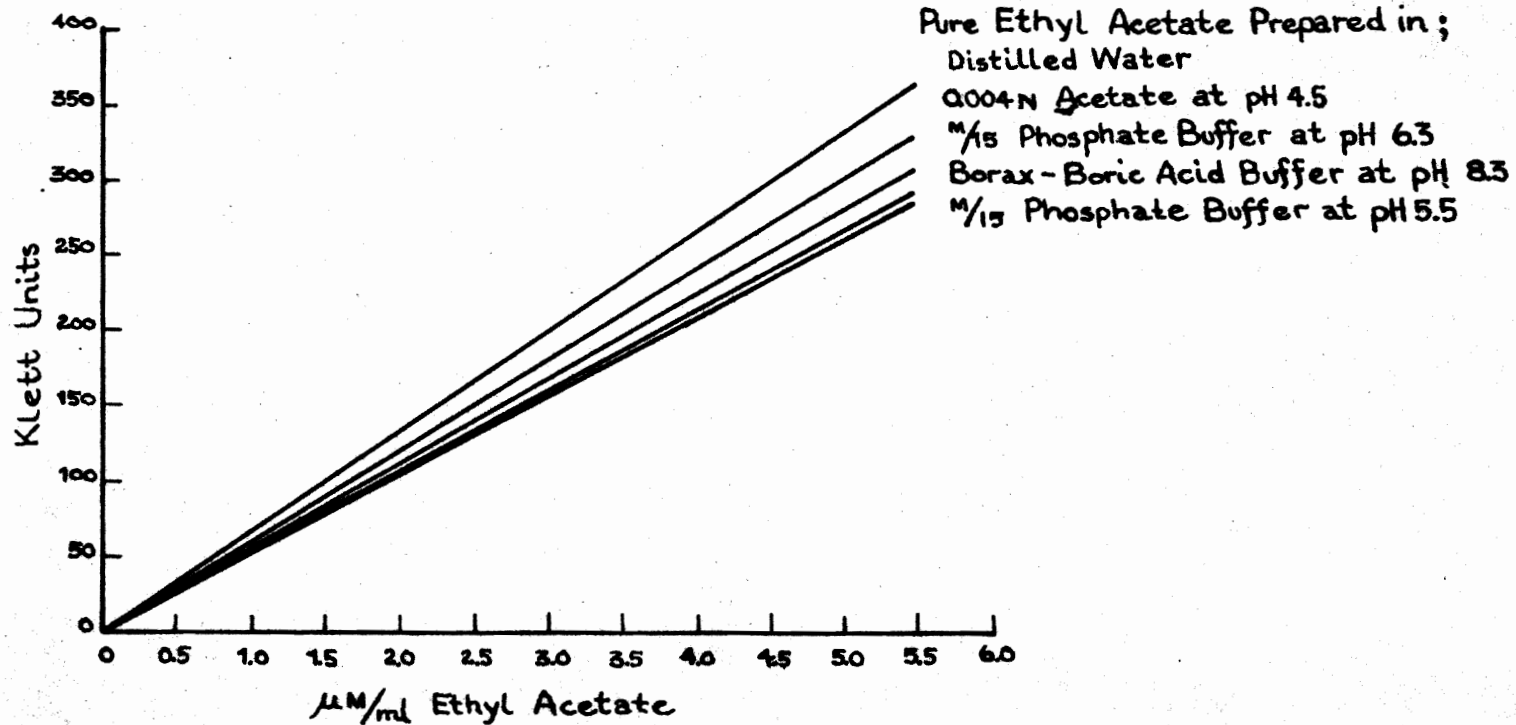procedure.

Studies of Ester Formation by Growing Cells of H. anomala 7

By the periodic addition of ethyl alcohol or acetaldehyde,
Larsen (1950) was able to demonstrate an increase in ethyl acetate
formation by a growing culture of H. anomala 7. He was unable to
demonstrate any increase by the addition of acetate.

The influence of a single addition of either ethyl alcohol,
acetaldehyde or acetate to a rapidly growing culture of this
organism was studied. The medium used was composed of the trace
and macro salts listed in Table I, 0.5 per cent asparagine, 0.2
per cent ammonium sulfate and glucose at a concentration of 0.015M.
It was adjusted to pH 5.5 and dispensed in 95 cc. amounts into
500 ml. Erlenmeyer flasks. These were sterilized at 15 pounds
pressure for 15 minutes. After cooling the flasks were inoculated
with a suspension of cells prepared as described in the section
Materials and Methods. The flasks were placed on a mechanical
shaker and shaken for 12 hours. At this time the proper amount
of ethyl alcohol, acetaldehyde, glucose or acetic acid contained
in 5 ml. of the complete basal medium was added to bring the
culture fluid to a concentration of 0.02 M with respect to the
compound involved. Aliquots were removed for determination of

Figure 1.

Comparison of Standard Curves For Ethyl Acetate Prepared in Various Buffers and Distilled Water

Pure Ethyl Acetate Prepared in ;
Distilled Water
0.004 N Acetate at pH 4.5
$^M/_{15}$ Phosphate Buffer at pH 6.3
Borax - Boric Acid Buffer at pH 8.3
$^M/_{15}$ Phosphate Buffer at pH 5.5

ester at 60 minute intervals. A graphic representation of the
results of the experiment including the control containing only
glucose is found in Figure 2. The single addition of acetalde-
hyde to a rapidly multiplying culture of the organism under study
resulted in a significant increase in ester concentration in the
culture liquid. A resultant drop in ester concentration after
the addition of ethyl alcohol or acetate was noted. The results
of this experiment suggest that the acetaldehyde concentration
is of immediate importance in determining the rate of synthesis
of ester by this organism.

Neuberg and Reinfurth (1918) reported experiments on alde-
hyde fixation with sodium sulfite in an effort to elucidate the
mechanism of alcoholic formation. Larsen (1950) has shown that
when acetaldehyde was added to a culture of H. anomala 7 an in-
crease in the production of ethyl acetate by this yeast results.
He suggested that acetaldehyde may be of immediate importance in
the synthesis of the ester. An attempt to demonstrate a change
in the amount of ester formed by a culture of this organism when
sodium bisulfite was added was made. A preliminary experiment
indicated that addition of sodium bisulfite at a concentration
of 0.001 M did not materially reduce the overall yield of cells
from a 24 hour aerated culture. Higher concentrations did show
a toxicity with a resulting decrease in cellular formation.

The basal medium containing the trace and macro salts listed
in Table I. plus asparagine and ammonium sulfate as nitrogen
sources, and 0.015 M glucose was used in this experiment. It

Figure 2.

The Effect of A Single Addition of Ethyl Alcohol, Acetaldehyde
and Acetate on the Synthesis of Ethyl Acetate by
Hansenula anomala 7

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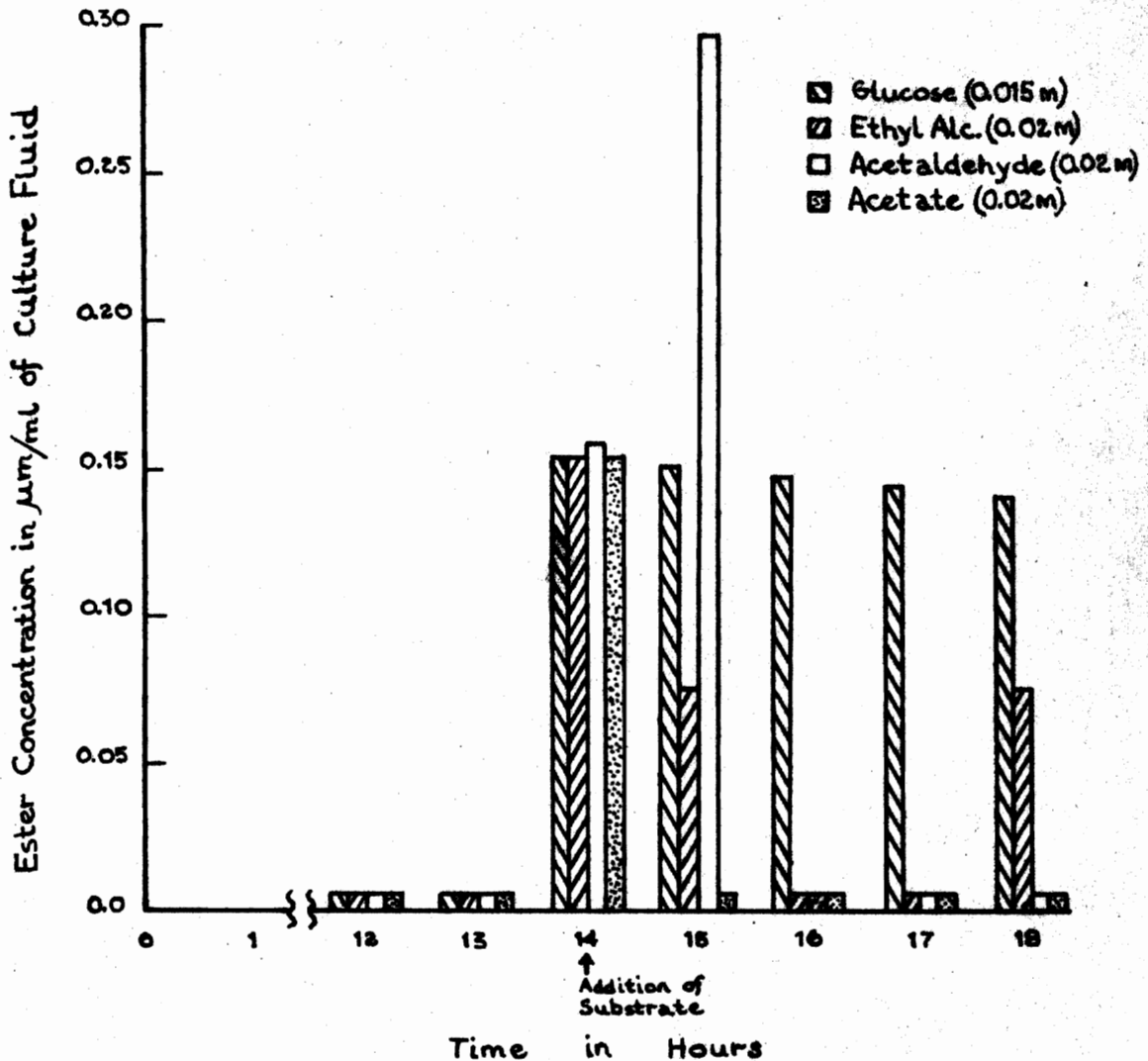
was adjusted to pH 5.5 and sterilized. Ninety ml. amounts were aseptically pipetted into sterile 500 ml. Erlenmeyer flasks and inoculated with H. anomala 7 cells. The flasks were placed on a mechanical shaker and agitated for a period of 12 hours. Aliquots were taken at 12, 13 and 14 hours and assayed for ester concentration. At the 14th hour of culture 5 ml. of the basal medium plus ethyl alcohol, acetaldehyde or sodium acetate were added. At the same time 5 ml. of a solution of sodium bisulfite in the basal medium were added to the test flasks. The added substrates were at such concentrations so as to bring the final concentration to 0.02 M.. The final concentration of bisulfite was 0.001 M.. The control flasks received 10 ml. of the complete basal medium. One hour later, the 15th hour of growth, samples were taken and ester concentrations determined in duplicate. The results are summarized in Table II. The concentration of ester noted was the

Table II

Flask Contents	Conc. in uM/ml of Ethyl Acetate
Glucose + Ethyl Alcohol	0.075
Glucose + Ethyl Alcohol - Sodium Bisulfite	<0.05
Glucose + Acetaldehyde	0.29
Glucose + Acetaldehyde - Sodium Bisulfite	0.15
Glucose + Acetate	<0.05
Glucose + Acetate - Sodium Bisulfite	<0.05

average of two determinations.

These data suggest again that acetaldehyde is of importance in the synthesis of ethyl acetate by H. anomala 7. This would seem to be in agreement with the observations made by Larsen (1950) and Peel (1951). Because of the relatively high toxicity of sodium bisulfite for cells of H. anomala 7, addition of larger quantities to growing cultures and the macro-recovery of ethyl acetate could not be made.

A study of the effect of the addition of propionaldehyde and isopropyl, n-propyl, n-butyl, isobutyl and t-butyl alcohols on the synthesis of ethyl acetate by H. anomala 7 was undertaken. The medium used in this experiment was composed of the trace and macro salts listed in Table I, o.5 per cent asparagine, 0.2 per cent ammonium sulfate and 0.02 M glucose. This medium was adjusted to a pH of 5.5 and dispensed into 2 liter suction flasks in 350 ml. amounts and sterilized. After cooling, the flasks were inoculated with cells washed from 24 hour stock agar slant cultures. The flasks were placed on mechanical shakers and agitated. After 12 hours of growth the flasks were connected with a forced air supply. The course of the affluent air was first through a column of loosely packed cotton, bubbled through a tube of distilled water and finally into the culture flask by means of a glass tube inserted through a rubber stopper which replaced the original cotton stopper. The glass tubing came to within an inch of the level of the culture fluid. This apparatus was sterilized prior to connection with the culture flasks.

Observation of the tube of distilled water served as a rough means for equating the flow of air through the individual flasks. The effluent air was taken out of the culture flask by means of the side arm and finally through a column containing approximately 200 gm. of charcoal. The charcoal in the column was previously washed with flowing stream and dried by forced air. This technique was similar to the one described by Larsen (1950) for the recovery of ethyl acetate from aerated and agitated cultures of ester-forming yeasts.

One ml. amounts of the alcohols or the aldehyde were added at each time interval to the appropriate flask. The flasks were under constant agitation and aeration. The schedule of addition was at 12, 24, 36, 48 and 60 hours. The total amounts of propionaldehyde and isopropyl, n-propyl, n-butyl, isobutyl and t-butyl alcohols added were approximately 0.07, 0.066, 0.066, 0.054, 0.054, and 0.054 M respectively.

The method used for the recovery of any possible esters formed from the above alcohols or aldehyde was a modification of that outlined by Shriner and Fuson(1940). After 72 hours of growth the liquid cultures were treated in two ways depending upon the boiling point of the ester expected. This was assuming that the esters formed would be, in the case of the alcohols, the acetate ester. In the case of the culture of $\underline{H}$. $\underline{anomala}$ 7 to which propionaldehyde was added any ester formed was assumed to have a boiling point greater than $100^{\circ}C$.

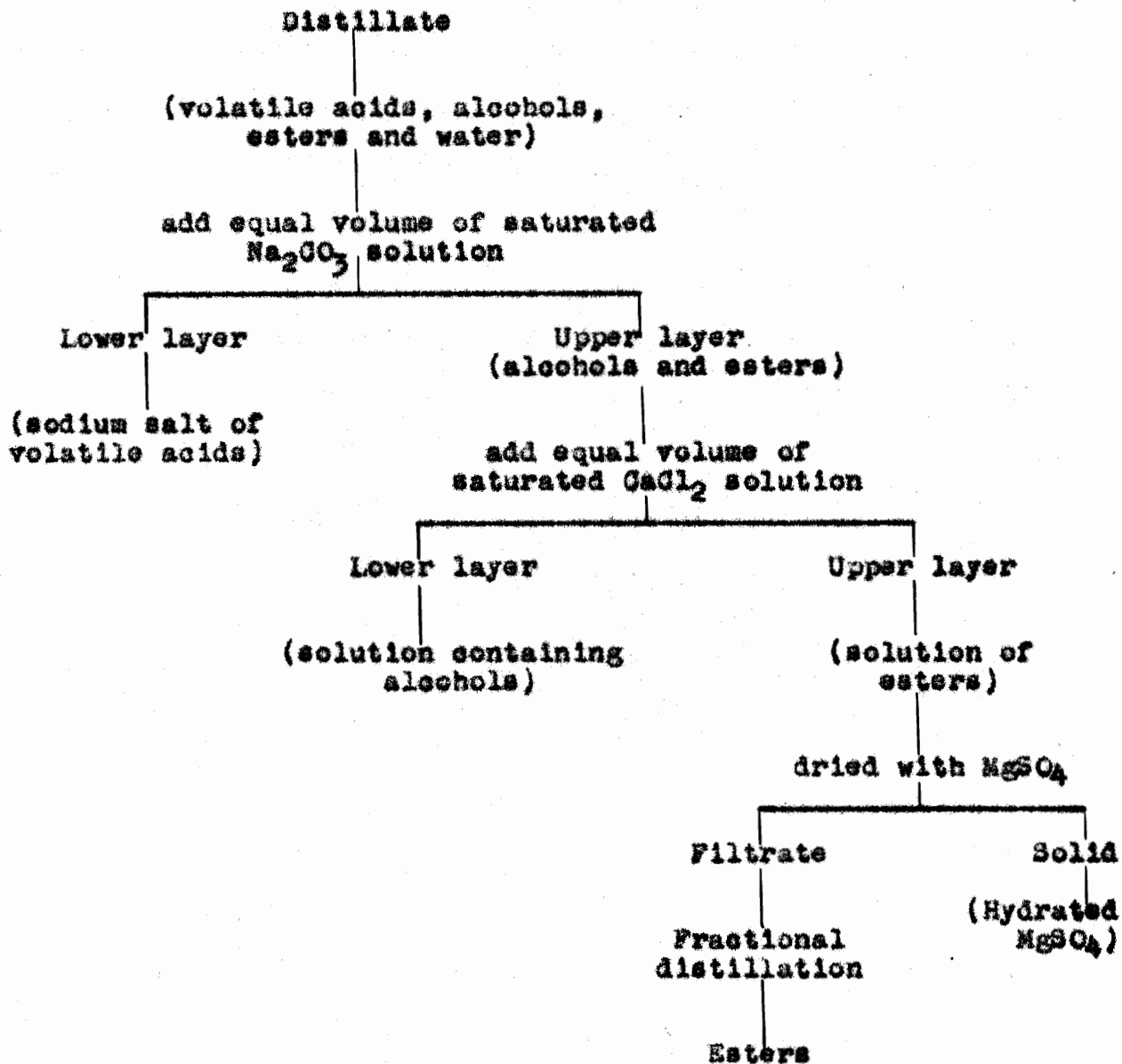The culture to which the isopropyl alcohol had been added

was rinsed into a liter Erlenmeyer flask with 100 ml. of water, boiling stones added, and connected with the charcoal column. The culture fluid was heated to boiling and the first 50 ml. of distillate recovered. This fraction was treated in a manner as outlined in Figure 3 taken in part from Shriner and Fuson (1940).

The cultures to which the other alcohols or propionaldehyde had been added were extracted with 100 ml. of ethyl ether and the ether soluble fraction separated. This solution was then used to extract the charcoal column. The solution taken from the column was composed of two phases, ether and water. The ether was distilled off at approximately $35^{\circ}C$. and the remaining solution treated as described in Figure 3.

The reliability of the procedure was checked by the addition of a known volume of ethyl acetate (2.0 ml.) to a distillation flask connected to a charcoal column. The procedures conducted were the same as those already described for the isolation of isopropyl acetate. An approximate recovery of 50 per cent on the basis of the volume of ethyl acetate added was accomplished.

The isolation of esters from cultures of H. anomala 7 to which propionaldehyde and higher alcohols were added was uniformly negative. The amount of growth as measured by determining the per cent packed cells was approximately the same for all cultures with the exception of that to which propionaldehyde was added. In this culture there appeared to be no increase in the amount of packed cells between the 12th and 72nd hour of growth.

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Figure 3

Separation of Esters from
Other Reaction Products

Distillate

(volatile acids, alcohols,
esters and water)

add equal volume of saturated
Na_2CO_3 solution

Lower layer

(sodium salt of
volatile acids)

Upper layer
(alcohols and esters)

add equal volume of
saturated $CaCl_2$ solution

Lower layer

(solution containing
alcohols)

Upper layer

(solution of
esters)

dried with $MgSO_4$

Filtrate

Fractional
distillation

Esters

Solid

(Hydrated
$MgSO_4$)

Studies of Ester Formation and Oxygen Uptake by Resting Cells

Oxygen uptake by resting cells of H. anomala 7 was deter-
mined in the Warburg apparatus. The concentration of ester in
the reaction mixture was determined after measurement of oxygen
uptake. The substrates studied were ethyl alcohol plus acetate
or acetaldehyde.

All tests were conducted in 20 ml. Warburg flasks with two
side arms. These flasks were shaken at approximately 90 oscil-
lations per minute in a water bath set at $30^{\circ}C$. The cells used
were from 24 hour stock agar slants. The cells were harvested
and washed in distilled water three times by centrifugation.
They were then suspended in a buffered salt solution composed
of the trace and macro salts listed in Table I. The pH of this
solution was adjusted to 4.5. Cell suspensions were aerated for
30 minutes at room temperature on a mechanical shaker. After
aeration the turbidity of the suspension was adjusted to a Klett
reading of 400. All substrates were dissolved in the buffered
salt solution at a pH of 4.5.

The flasks used for obtaining data on the oxygen uptake and
subsequent ester formation by resting cells contained 1.0 ml. of
buffered salt-ethyl alcohol solution and 1.0 ml. of cell suspen-
sion in the main chamber. The center well contained 0.4 ml. of
15 per cent potassium hydroxide. One of the side arms contained
0.5 ml. of a buffered salt-acetaldehyde or acetate solution. After
mixing, the final concentrations of the substrates were ethyl
alcohol, 0.2 N; sodium acetate, 0.008 N; and acetaldehyde, 0.01 M.

This acetate concentration was the same as that at which Peel (1951) reported a 50 per cent increase in the amount of ethyl acetate formed in the presence of 0.2 M ethyl alcohol. The acetaldehyde concentration was the same as that at which he obtained an increased ester synthesis by washed cells in the presence of ethyl alcohol.

After the addition of the components of the reaction to the flasks and attachment to the manometers, an equilibration period of 10 minutes in the water bath was allotted before closure of the system. Several readings at 15 minute intervals were made prior to tipping of the side arm which contained the second substrate or buffered salt solution. After completion of the observation period the flasks were detached and the contents removed. Duplicate ester determinations were made with this suspension.

The results of this type of experiment are shown in Figure 4. The final ethyl acetate concentrations of the various reaction mixtures were: ethyl alcohol plus buffered salt solution, 0.000 uM/ml.; ethyl alcohol plus acetaldehyde, 0.00 uM/ml.; ethyl alcohol plus acetate, 0.125 uM/ml.; and, buffered salt solution, 0.00 uM/ml. It would appear that the addition of 0.008 M acetate to 0.2 M ethyl alcohol did not increase the oxygen uptake by H. anomala 7 cells while it did significantly increase the synthesis of ethyl acetate. The apparent decrease in oxygen uptake by this yeast in the presence of a substrate composed of ethyl alcohol plus acetaldehyde was probably due to the great volatility of the

Figure 4.

Oxygen Uptake by H. anomala 7 in the Presence of Ethyl Alcohol and Acetate or Acetaldehyde
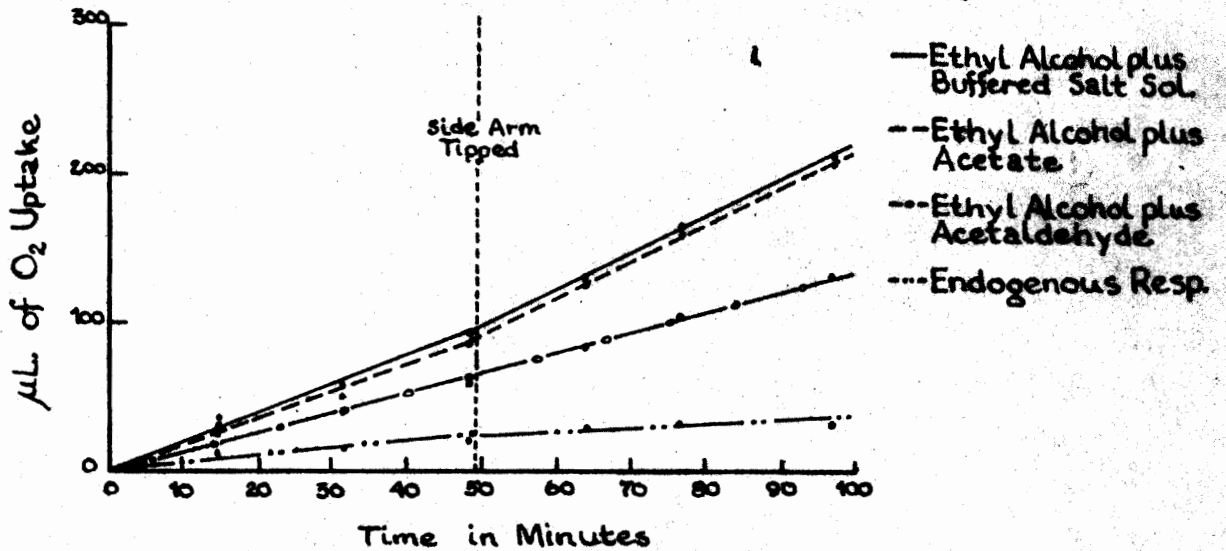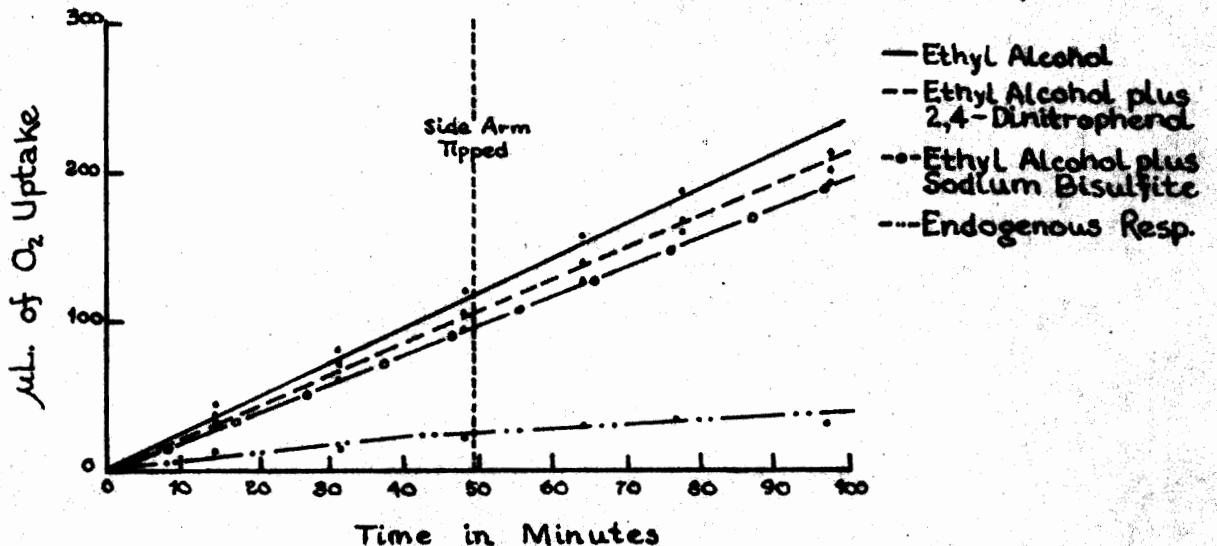

Figure 5.

Oxygen Uptake by H. anomala 7 in the Presence of Ethyl Alcohol and Sodium Bisulfite or 2,4-Dinitrophenol

aldehyde. The addition of 0.01 M acetaldehyde to 0.2 M ethyl
alcohol did not produce any measurable ethyl acetate.

Hotchkiss (1944) found that 2,4-dinitrophenol (DNP) in-
hibited aerobic phosphorylations by staphylococci without in-
fluencing oxygen uptake. Peel (1951) has suggested that the
synthesis of ethyl acetate by H. anomala may proceed by means
of the formation of a labile acetyl derivative which reacts
with ethyl alcohol to produce the ester. He further suggested
that this postulated acetyl derivative may be acetyl phosphate.
The effect of 2.4×10^{-4} M 2,4-dinitrophenol and 0.001 M sodium
bisulfite on washed cells of H. anomala 7 in the presence of
ethyl alcohol was studied. The same techniques were used in
this experimental procedure as were used in the study of the
effects of the addition of acetate and acetaldehyde to ethyl
alcohol. The DNP and the bisulfite were added to a side arm
in 0.5 ml. quantities at such concentration as to bring the
final concentration of the reaction mixture up to that described
above. Ester determinations were made on the contents of the
flasks after measurement of oxygen uptake.

Figure 5 summarizes the data obtained in this study.
Neither DNP nor bisulfite appeared to influence the oxygen up-
take by H. anomala 7 in the presence of ethyl alcohol. No meas-
urable ester was formed either in the presence or absence of
these compounds.

With the concentration of cells used in this group of
studies, it would appear that added acetate (0.008 N) is

necessary for the synthesis of ethyl acetate by H. anomala 7.

Studies of Ester Synthesis in the Presence of Carbon-14 Methyl Labelled Sodium Acetate.

Peel (1951) reported that the addition of acetate at a concentration of 0.008 M to 0.2 M ethyl alcohol resulted in a 50 per cent increase in ethyl acetate synthesis by washed cells of H. anomala. He suggested that isotope studies might lead to information concerning whether added acetate is incorporated into ethyl acetate or not.

A generous gift by Dr. Leo T. Samuels of a small quantity of methyl labelled sodium acetate made this study possible. The medium used was composed of 0.1 per cent yeast extract, 0.1 per cent potassium acid phosphate, 0.5 per cent peptone and 0.008 M sodium acetate. Two different ethyl alcohol concentrations were employed, 0.1 M and 0.2 M. Twenty seven ml. of the freshly prepared medium at pH 4.6 were added to round bottom distillation flasks and the proper amounts of ethyl alcohol added. Two ml. of a solution of carbon-14 methyl labelled sodium acetate containing 23.7 ugm/ml were added. The main opening of these flasks was closed with a one hole rubber stopper which contained a length of glass tubing. The end of the tubing which projected into the flask was drawn out to a fine capillary. The glass tube was connected by rubber tubing to a 4000 ml. Erlenmeyer flask which contained pure oxygen. This arrangement would afford a relatively constant supply of oxygen to the growing yeast.

The oxygen reservoir was inverted in a large container of water so that water replaced the oxygen as it was used up. Diffusion of gases between the oxygen reservoir and the culture flask was reduced by the capillary. The side arm of the distillation flask was connected to a 75 ml. test tube by means of a rubber stopper. This test tube contained 10 ml. of 15 per cent potassium hydroxide which would absorb the carbon dioxide formed during growth.

The flasks were inoculated with 1.0 ml. of a suspension of cells washed from 48 hour stock agar slant cultures and placed on a mechanical shaker. The conditions of incubation were at room temperature for 24 hours.

After 24 hours. of growth the flasks were removed and disconnected from the oxygen supply. The carbon dioxide absorption tubes were disconnected and stoppered. By addition of phenolphthalein and sodium hydroxide, the pH of the contents of the distillation flasks was brought to approximately 8.0. The flasks were heated and the first few milliliters of distillate collected in 10 ml. volumetric flasks which were chilled in an ice bath. These distillates were each diluted to exactly 10 ml. with distilled water. The ethyl acetate concentration of these solutions was determined and was found in each case to be greater than 50 uM/ml.

One ml. quantities of each of the above solutions of ethyl acetate were mixed with 2.0 ml. of alkaline hydroxylamine. One ml. of each of these solutions was pipetted into stainless steel cups. One ml. of each of the liquid cultures after distillation

and 1.0 ml. of the potassium hydroxide solutions were placed in similar cups. The results of the counting of these samples are found in Table III. Background counts were determined before and after counting of the samples. The equipment used was described previously under Experimental Methods and Materials.

Table III

Formation of Ethyl Acetate by a Growing Culture of
H. anomala 7 in the Presence of Labelled
Acetate

Composition of Medium	Alkaline Hydro-xylamine-Ester Solution	Culture Liquid	Potassium Hydroxide Solution
Ethyl alcohol (o.2 M) plus labelled and non-labelled acetate (0.008 M)	33 CPM	35 CPM	49 CPM
Ethyl alcohol (0.1 M) plus labelled and non-labelled acetate (0.008 M)	32 CPM	35 CPM	54 CPM

Note: Background count = 33 counts per minute.

The data suggest that the majority of the added acetate was oxidized to carbon dioxide and water. No increase over the background count in counts per minute was observed in the ester produced by a growing culture of H. anomala 7, in the presence of radioactive acetate.

Cell Free Enzyme Studies

Various procedures were used in an attempt to obtain a cell free enzyme preparation from cells of H. anomala 7. The cells used were obtained as previously described. The techniques involved were acetone drying and extraction with M/15 phosphate buffer at pH 6.3; treatment with toluene and extraction; passage of a yeast cell-ground glass suspension in buffer between rotating glass surfaces; alternate freezing and thawing with extraction at 6°C.; drying of the yeast cells from the frozen state with subsequent grinding of the powder in a ball mill, and extraction at 6°C. or room temperature; and, grinding of a cell paste in a ball mill at 6°C. followed by extraction with M/15 phosphate buffer at pH 6.3 or with distilled water in the cold. The suspensions obtained by the above methods were centrifuged at high speed in the cold and the supernatant solutions tested for enzyme activity.

The procedure of testing for enzyme activity consisted of the addition of 1.0 ml. of the cell free enzyme preparation to a mixture of 1.0 ml. each of ethyl alcohol and sodium acetate. These substrates were prepared at concentrations of 0.02 M in phosphate buffer at pH 6.3. The system was incubated at 30°C. for 60 minutes at which time ester determinations were made.

The only procedures by which active cell free preparations were obtained were those involving grinding of the yeast cell paste in the ball mill followed by extraction with either phosphate buffer or distilled water at 6°C., or that involving

drying of the cells from the frozen state, grinding of the dried powder, followed by extraction in the cold with phosphate buffer. The latter method gave less active preparations. The former procedure will be described in more detail.

Washed packed cells of H. anomala 7 were placed in a 250 ml. centrifuge bottle and glass marbles added. This bottle was placed on the rollers of a ball mill and rotated for 24 hours at approximately $6^{\circ}C$. At this time an equal volume of cold M/15 phosphate buffer at pH 6.3 was added. The bottle was returned to the ball mill and rotated an additional 12 hours. The suspension was removed and centrifuged at high speed in the cold for 60 minutes. The supernatant was tested for activity with 0.02 M ethyl alcohol at pH 6.3 alone, or with the alcohol plus 0.02 M sodium acetate at the same pH. The conditions of incubation were at $30^{\circ}C$. for 60 minutes. Duplicate ester determinations were made. The results were; ethyl alcohol plus acetate yielded 0.2 uM of ester per ml. of reaction mixture, and ethyl alcohol plus buffer yielded 0.2 uM of ester per ml. of reaction mixture. The results of a similar experiment in which aliquots were removed at 10 minute intervals are shown in Figure 6. All components of the system were added to 250 ml. Erlenmeyer flasks at 10 times the volume of the previous experiment.

Ethyl acetate synthesis by cell free preparations from H. anomala 7 in the presence of 0.02 M ethyl alcohol does not appear to be influenced by the addition of 0.02 M sodium acetate. This finding was in agreement with that of Larsen's (1950) who

Figure 6.

Activity of Cell Free Enzyme
Preparations From H. anomala 7

36
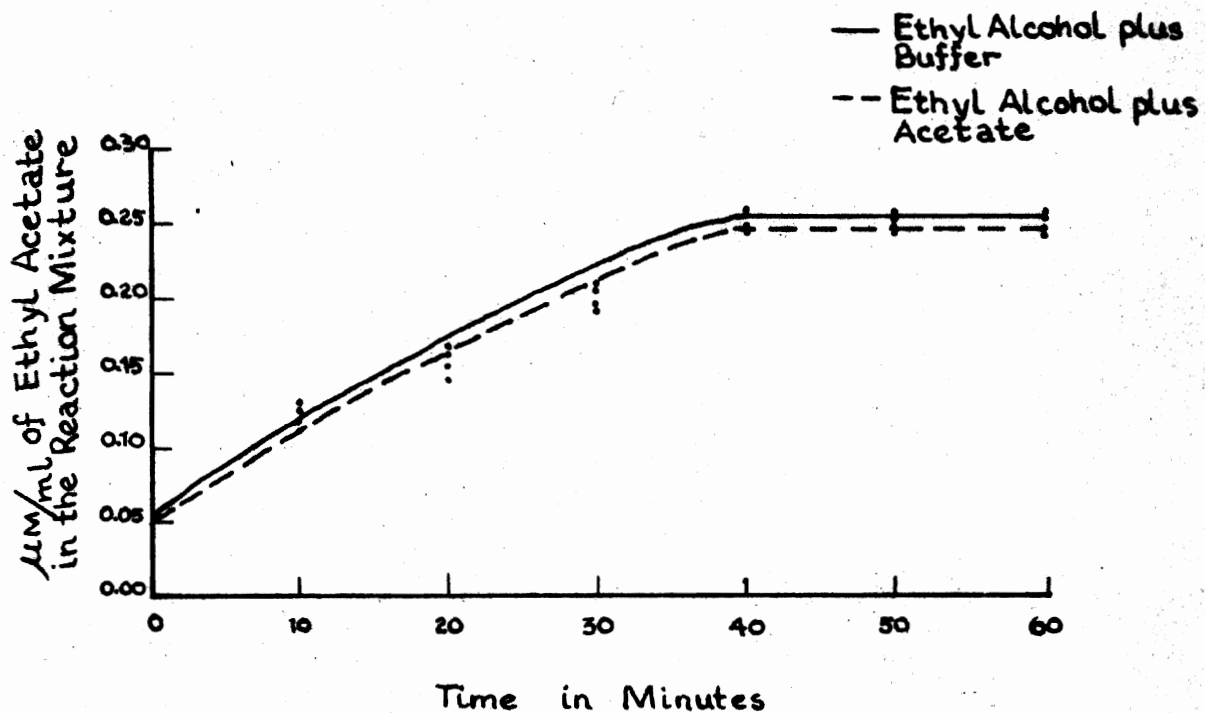
found that added acetate did not increase the formation of ethyl acetate by growing cultures of H. anomala 7.

Attempts to increase the activity of the enzyme preparations by various means were always followed by complete loss of activity. Storage of an active preparation at $6^{o}C$. for 24 hours resulted in complete loss of measurable activity. To this inactive preparation the addition of 0.3 uM of adenosine triphosphate or 3.0 uM of diphosphopyridine nucleotide with 0.004 M nicotinamide did not result in any return of activity.

DISCUSSION

The results of the experiments in which acetaldehyde was added would seem to indicate that acetaldehyde is an important intermediate in the synthesis of ethyl acetate by H. anomala 7. The reduction in the concentration of ester in acetaldehyde medium after the addition of sodium bisulfite would appear to justify this statement. This reduction of the concentration of ester is suggested to be due to prevention of the aldehyde from reacting to form ethyl acetate by complex formation of the acetaldehyde with bisulfite. The abrupt drop in ester concentration after introduction of the bisulfite may be due to increased esterase activity by the multiplying yeast cells. This increased esterase activity would be initiated by the sudden increase in ester concentration after the addition of the acetaldehyde. Also increased esterase activity coupled with the supposed blockage of ester synthesis caused by the addition of bisulfite would ultimately result in a reduced ester concentration.

An explanation of the seemingly conflicting results concerning the effects on ester synthesis by the addition of various concentrations of acetate was postulated by Peel (1951). He suggested that the marked stimulation of ester production followed by no effect or inhibition, occuring when the acetate concentration was increased, may be due to an alteration of the external pH required to give optimal internal conditions for

ester formation from ethyl alcohol alone.

The data presented in the experiments with labelled acet-
ate would suggest that the acid moiety of the ester is not derived
from added acetate. Added acetate appeared to be rapidly oxidized
to carbon dioxide and water. Because of the low concentration
of labelled acetate necessarily used, the experiment should be
repeated using much higher concentrations of radioactive acetate.

The higher alcohols used in an attempt to induce the yeast
to form other esters were oxidized and utilized as carbon sources
for cellular multiplication. This would suggest that the enzymes
involved were more or less specific in that ethyl alcohol was
the only alcohol from which ester was formed.

There are numerous problems involved in obtaining a cell
free enzyme preparation which would carry out the synthesis of
ethyl acetate in the presence of a suitable substrate. Peel
(1951) has reported that storage of freshly washed cells for
24 hours at $1^{\circ}C$. results in a 50 to 75 per cent loss in en-
zymatic activity. The instability of cell free preparations
would necessitate extensive studies concerning the stability
of activity. The possibility that the extremely unstable
nature of these preparations was due to destruction of a certain
cofactor or co factors is postulated.

SUMMARY

The single addition of acetaldehyde to a rapidly multiplying culture of H. anomala 7 resulted in a significant increase in the synthesis of ethyl acetate. After addition of ethyl alcohol or acetate, a drop in the concentration of ester was noted.

A reduction in the concentration of ethyl acetate in a rapidly growing culture of this yeast was found when sodium bisulfite, 0.001 M, was added to a medium containing acetaldehyde or ethyl alcohol.

The isolation of esters from cultures of H. anomala 7 to which propionaldehyde and isopropyl, n-propyl, isobutyl, n-butyl and t-butyl alcohols were added was uniformly negative. The yeast was able to utilize the alcohols for growth. The propionaldehyde appeared to be toxic at the concentration added.

While addition of 0.008 M acetate did not increase the oxygen uptake by resting cells when in the presence of 0.2 M ethyl alcohol, its presence brought about an increased synthesis of ethyl acetate by resting cells of this yeast.

Neither 2,4-dinitrophenol nor sodium bisulfite appeared to influence the oxygen uptake by resting cells of H. anomala 7.

The data obtained using radioactive sodium acetate suggested that the majority of added acetate was rapidly oxidized to carbon dioxide and water. No measurable increase over the background count in counts per minute was observed in the ethyl acetate

produced by H. anomala 7 when in the presence of labelled acetate.

Isolation of a cell free preparation obtained from cells of H. anomala 7 was noted. These preparations were low in enzyme activity and an increased activity could not be attained by the addition of 0.3 uM of adenosine triphosphate or 3.0 uM of diphosphopyridine nucleotide. Acetate concentration at 0.02 M did not effect an increase in ethyl acetate synthesis with the cell free enzyme preparation.

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