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# The biochemistry of sluggish butyl-acetonic fermentations

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THE BIOCHEMISTRY OF SLUGGISH BUTYL-ACETONIC  
FERMENTATIONS

BY

DONALD F. STARR

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A Thesis Submitted to the Graduate Faculty  
for the degree

DOCTOR OF PHILOSOPHY

Major Subject Bio-physical Chemistry

Approved:

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1933

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TABLE OF CONTENTS

I.	ACKNOWLEDGEMENT	2
II.	INTRODUCTION	4
III.	THE INVESTIGATION	14
	A. Cultures Studied	14
	B. General Methods	15
	C. Isolation and Culture of the Sluggish Principle	17
	D. Filtration Studies	22
	E. Immunization of Cultures Toward the Action of the Sluggish Principle	31
	F. Properties of the Filtrate	34
IV.	SUMMARY AND CONCLUSIONS	79
V.	REFERENCES CITED	82

## INTRODUCTION

The butyl-acetonic fermentation process as normally carried out on an industrial scale (17)(33)(40)(41) utilizes cereals, potatoes, molasses, or other sources of carbohydrate in the production of n-butanol, acetone, ethanal, carbon dioxide and hydrogen. The use of low grade corn is common industrial practice and a normal fermentation (25) converts three pounds of starch into one pound of solvents and over one and one-half pounds of gases in about forty-eight hours. Gabriel (16) reported the Commercial Solvents Corporation had a total of 148 fermenters of 50,000 gallons each in operation in two plants. Combined they were capable of using more than 30,000 bushels of corn per day. To convert this amount of raw material into solvents, about 600 tons of coal and 15 million gallons of water were required daily. The solvent production for that year was over 85 million pounds with a peak production (19) of 120 million pounds in 1929.

The normal course of the fermentation undergoes three phases (42)(43). In the first or initial stage there is vigorous bacterial reproduction and acid formation. The second stage occurs after the maximum acidity of the mash has been reached and there is a rather rapid



decrease of acidity to a minimum. In the last or third part of the fermentation there is a gradual rise of the acidity of the mash to a fairly constant level.

Peterson and Fred (36) have published a complete summary of all the changes taking place in the mash during the three stages. The starch is being hydrolysed rapidly during the first part to produce a maximum of reducing sugars at about the midpoint of the fermentation. As the acidity drops there are formed neutral products, n-butanol, acetone, and ethanol, and the bacterial reproduction ceases. The evolution of gases reaches a maximum and pushes the insoluble part of the corn to the top of the medium forming a characteristic head (31) (for illustration see Fig.3). During the gradual rise of acidity in the last part of the fermentation there is also gradual increase in solvents and an increase in the number of spores (31).

The outstanding pioneers in the investigation concerning the bacterial production of n-butanol include Pasteur (34), Fitz (7 - 11), Grimbart (22), Beijerinck (1,2), Bredemann(3), Fernbach (5,6), and Weizmann (50). There are many other workers who have contributed to the information available but to each of the above may be attributed some definite step in advance.

By dilution methods Fitz (10) prepared cultures which he called Bacillus butylicus and which he believed to be pure. The bacteria were observed in a counting chamber and then diluted until each five drops or ten drops would contain one bacterium. Fifty flasks were

inoculated with one drop each and about five or ten flasks showed growth. From the fermentation of a variety of substrates he identified n-butanol by its boiling point and by the boiling point of n-butyl iodide. Ethyl alcohol was obtained in small amounts. If acetone were also produced, the method of analysis would not have shown it.

Grimbert (22) conducted his fermentations with an organism which he believed to be different from those of Pasteur, Van Tieghem, Fitz and Pedrix because of different cultural and physiological characteristics. He named the bacteria Bacillus orthobutylicus. The fermentations were performed with and without chalk, using various substrates, and in all cases there was more acid and less n-butanol where appreciable quantities of chalk were present.

Beijerinck (1) proposed a new genus, Granulobacter, for the butyl organisms and related types. The Gr. saccharobutyricum was similar to Fitz culture while Gr. butylicum was able to produce iso-propyl alcohol with the n-butyl alcohol. Gr. lactobutyricum was able to ferment lactate solutions and Gr. polyoxyza was identical to the facultative Clostridium polyoxyza of Prazmowski. Later Beijerinck and Van Delden (2) added another species, Gr. pectinovorum, which was the active organism in retting flax and possessed the ability to attack pectins.

Several strains of the butyric acid and butyl alcohol bacteria, previously reported in the literature

under various names, were collected and studied by Bredemann.

(3) In 1909 an extensive report was published by him comparing all of the strains studied; eleven strains were classed as belonging to the same species and renamed Bacillus amylobacter A.M. et Bredemann while eighteen other strains were considered as probable members of his species. He attributed variations of the cultures to the conditions of cultivation.

Fernbach and Strange (5) (6) obtained patents covering a commercial process for the production of acetone and butanol from various carbohydrate materials by means of a culture of "bacillus of the type of Fitz". Their patents included provisions for adding degraded yeast as nutrient and conducting the fermentation in the absence of air. The pressure was reduced and the volatile products distilled off as they were formed. Fernbach was the first to report the simultaneous production of acetone and n-butanol.

The Fernbach process was used on a commercial scale for three years. During the World War, Weizmann (50), and a former colleague of Fernbach's developed a culture which he claimed was unlike Fernbach's, and which gave better commercial scale fermentation than did Fernbach's. In the plants operated under the control of the British Admiralty, the Weizmann culture was employed, and the United States Court of Appeals (18) has held that the

cultures now used in the industry in this country are of the Weizmann type.

There are many accounts in the literature concerning various types of abnormal fermentations. In some of the very early work with the butylic fermentation Fitz (10) described cultures which had lost the ability to produce n-butanol due, he believed, to high oxygen tension, too high pasteurization temperature or to high incubating temperature, 45°C. or more.

Speakmann (42) reported slow fermentations and gave gas and acidity curves characteristic of this type of fermentation but he did not offer an explanation for the abnormality.

Several investigators (47) (12) (44) have described harmful bacterial contamination in their fermentations. Organisms producing lactic acid were most frequently isolated from the contaminated fermentation. Apparently the butyl organisms converted the starch to glucose only to find the lactic acid organisms competing for the supply of sugar and producing an acidity which finally halted the growth of both species. In the cases where inhibition was due only to the high acidity, neutralization of the mash allowed the formation of solvents to take place, since moderate amounts of calcium lactate can be converted to the normal end-products.

The effect of lactic acid producing contaminants was studied in detail by Fred, Peterson and Mulvania (13). They reported that the most serious contaminant was Lactobacillus leichmanni which they considered to be the same as

the Bacillus volutans studied by Thaysen (47) and later by Speakman and Phillips (44). The evidence presented shows that the toxicity of the lactic acid contamination was due to the acid produced. Filtrates from their sluggish fermentations were not toxic after being neutralized. They were able to grow the cultures separated only by a collodion bag (32) and found that there was no toxic material diffusing through the bag excepting the lactic acid, which was toxic when present in sufficient quantity.

The butyl organisms are capable of fermenting a varied group of carbohydrates, but the acid production is also varied. Robinson (37) made a careful study of several substrates by following the acidity curves during the fermentations. In general the compounds which he studied fell into three groups; first, the group which produced acidity curves similar in shape to those obtained in a normal fermentation of corn mash; these included glucose, fructose, mannose, sucrose, starch and biologically prepared dextrin; second, the group which produced abnormally high acidity curves having no "acidity-break"; these included galactose, xylose, arabinose, lactose, raffinose, melezitose, inulin, mannitol, and dextrin produced by acid hydrolysis of starch; third, the group including those which were not fermented.

Several investigators (6) (35) (38) (45) (53) have studied the relationship between the nitrogen and carbon metabolism of the butylic bacteria, and it is agreed that a normal yield of solvents can not be produced if a protein deficiency exists. Weinstein and Rettger (49) have made a study of the n-butanol fermentation in the presence of several different types of protein and have concluded that zein is especially suitable for a normal fermentation, giving a full yield of neutral products in their normal ratio.

In the industrial production of n-butanol by fermentation it has been found that the most vigorous fermentations are obtained by using about the sixth subculture from spores, because for some reason the cultures which are transferred continuously beyond the sixth subculture are not good solvent producers. Sluggish fermentations are usually encountered on the ninth to twelfth subculture from spores, and Gill (20) mentions that even the fourth or fifth transfer in tubes gives sluggish fermentations.

Freshly isolated cultures are usually found to be slow fermenters, and culturing in the laboratory on corn mash or similar media for one or two years is often necessary to produce a culture of maximum vigor in producing solvents. Weyer and Rettger (54) have treated cultures

for a period of nearly two years with a gradual increase in the acetone yields.

In the Weizmann patent (50) there has been described a process for producing vigorous cultures for commercial use which included 100 or 150 cycles of inoculation, heat shock and incubation, requiring four or five days for each cycle or in all from one two two years.

Thaysen (47) has mentioned that lactic acid producing contaminants are serious but that occasionally abnormal acidity curves and slow fermentations were in practice which were not due to any bacterial infections. This type of fermentation has been more completely described by D.A.Legg (27). He divided the abnormal fermentations into two groups; the contaminated fermentation as described above, and the sluggish fermentation, characterized by a prolonged acidity peak, decreased yields of solvents, incomplete utilization of fermentable carbohydrate and slow fermentation. Legg states that : "The phenomenon occurs intermittently and for no apparent reasons. It is usually epidemic in character and of sudden incidence.

"When sluggish fermentation is once noticed in one or more vessels employed in a butyl-acetonic fermentation plant, within about twenty-four hours it will frequently become apparent in hundreds of disconnected vessels of various sizes, including laboratory cultures.

Even cultures which had been stored for years in sealed tubes in spore form, when transferred to and grown in sterile mash are not immune from sluggishness during a plant epidemic period."

It was found that a small portion of a sluggish fermentation coming in contact with normal fermentations caused sluggishness to appear in them. The mash from sluggish fermentation could be filtered through a Berkefeld or similar bacterial filter to remove the bacterial cells and still the filtrate contained the sluggish principle. The filtrate has been diluted a million times (27) and the resultant dilution frequently found to induce sluggishness in a normally fermenting mash.

The sluggish principle has been maintained by transferring a drop of the filtrate from a sluggish culture to a fresh mash and then filtering the culture after sluggishness has occurred. This process can be carried on indefinitely, showing that the process is truly a propagation of the sluggish principle and not merely a dilution of it. Legg explains that, " On the basis of these facts and on others not reported here, it would appear that sluggish fermentation is caused by a living organism of ultra-microscopic dimensions. If this is the case it may be either a saprophyte living in association with the butyl-acetonic organism, or it may be a true parasite or bacteriophage."



The Commercial Solvents Corporation experienced a severe epidemic (16)(19)(27) of sluggishness during the year 1923 which cut their production in about half for that year. Legg's patent covers the process for producing immune cultures and after the introduction of the more resistant strains the yields were reported to be generally higher and no more periods of sluggishness were encountered.

A toxic filtrate, similar to the one described by Legg, was accidentally obtained in this laboratory in 1931 from a freshly isolated culture from wheat. With the isolation of the toxic filtrate it was possible to study further the properties of the material described by Legg (27).

The purpose of the work here reported may be outlined briefly as follows :

1. To study the properties of the sluggish principle.
2. To study the biochemistry of the sluggish fermentation.
3. To study possible methods for eliminating sluggishness.

## THE INVESTIGATION

### Cultures Studied

Fifteen strains of butyl organisms have been studied during these experiments and except for the strains isolated in this laboratory the cultures are designated as they were when received. Two strains were obtained from the American Type Culture Collection, Clostridium acetoniigenum No.862 and Clostridium butyricum No.824.

Four strains studied and reported in the literature by Rettger and others were obtained from C.H.Werkman of the Department of Bacteriology, Iowa State College. These are strains SO, R, I, and B. Several of Fernbach's cultures were studied and designated as 12A, 12B, FBB, Fl, 101, A211, and M9. A strain was isolated from wheat and was named W3; W36 is a modification of W3. All of these strains probably belong to the species called Clostridium acetobutylicum by McCoy, Fred, Peterson and Hastings (31). Two other species, Flavobacterium suaveolens and Escherichia coli have been used and were obtained through the courtesy of M. Levine, Department of Bacteriology, Iowa State College.

### General Methods

The spores of the stock cultures were kept on sterile soil containing about five percent  $\text{CaCO}_3$  or sterile sea sand with one percent  $\text{CaCO}_3$ . During these experiments, as far as it was possible to do so, the cultures for study were always directly derived from the same spore cultures in order to have the test cultures remain as constant as possible during the study. Tubes inoculated with spores were always heat shocked two minutes in a boiling water bath.

The cultures were grown in ten-inch fermentation tubes containing 20 cc. of 5% corn mash. The transfers were made with wide tip, 3 to 5 cc. pipettes wrapped in tissue paper and sterilized. The inoculation ratios used were from 5% to 10% in tubes. Except where stated, the third to sixth subculture from spores was used for testing purposes.

Sluggish fermentations have high prolonged acidity peaks and do not produce the characteristic heads in corn mash. The solvent yields are usually lower than in normal fermentations and the final acidities are frequently higher. The simplest method of detecting the presence of the sluggish principle was to observe the head produced at 20, 24, and 40 hours and compare with the appearance of a normal fermentation.

The final acidities and solvent yields may be determined as checks on the appearance of the fermentation. The most reliable method of noting the presence of the sluggish principle is to follow the titratable acidity curve. It should be possible to obtain a sharp differentiation between the sluggish and normal fermentation from the titratable acidity in corn mash at about thirty hours after inoculation.

Microscopic observation has been reported to be useful in detecting some types of sluggish fermentations but no special differences have been noted during this study which would serve as a reliable check on the presence of the sluggish principle.

The Isolation and Culture of the Filterable Sluggish Principle

Apparently there is not the same opportunity for epidemics of sluggishness in an experimental laboratory that exist in a laboratory connected with a large fermentation plant, since there has been but one sluggish epidemic during two years work in this laboratory and that was with the cultures that were just being started for study.

The sluggish principle has not been widely encountered among our materials. There is considerable difficulty in isolating toxic filtrates from laboratory cultures that are handled and transferred under aseptic conditions and from cultures obtained from spores subjected to heat shock. The filtrate used in this laboratory was obtained from a new culture isolated from wheat and designated as W3. The culture had been carried in a mash acidified with 0.2% butyric acid and the sour mash filtered after fermentation had ceased. This toxic filtrate could be inoculated into other susceptible cultures and more of the toxic filtrate obtained. The cultures which were more susceptible appeared to produce filtrates which were a little more virulent. The sluggish principle can be carried by inoculating any susceptible culture and allowing the fermentation to be completed, and the mash when

filtered can be used again to inoculate a susceptible culture. No specificity between filtrates produced from cultures in this laboratory for any particular strains of susceptible cultures has been noted. That is, a particular filtrate may appear more toxic than some other one, but it will be more virulent for all of our susceptible cultures.

Several strains of butyl organisms have been subjected to the action of a toxic filtrate and the observations recorded in Table I. All tests were run in duplicate and where the heads appeared the same only one observation has been listed but where they had different appearance both observations are given.

The results in Table I may be interpreted as showing all of the strains susceptible except W36, FBB, and Cl. acetoniogenum. The Cl. acetoniogenum and W36 are considered resistant on the basis of their acidities, FBB is considered resistant on the basis of the heading and the acidity although one of the filtrate tubes is high in acid. Several of the controls in this group are abnormally high in acid. In general the control tubes are more regular than the ones in this group. This group of filtrate tubes is a fair representation of the variability encountered in filtrate tubes.

Several abnormal fermentations have been studied in attempts to isolate toxic filtrate but all of these attempts yielded negative results.

Four strains, W36, 101, FBB, and A211 were transferred for five consecutive transfers in mash acidified in varying amounts, with 0.2% to 0.6% of butyric acid but no toxic filtrates were obtained. However the immune strains FBB and W36 were also shown to be more resistant to acid.

At various times during this study, flasks of strain FBB were obtained which had received amounts of lactic, butyric, acetic and formic acids sufficient to cause a "sick" culture but in no case was a toxic filtrate isolated.

Strain W3 after having been purified by plating, was subcultured and a spore culture put on sterile soil, but could not be made to produce a toxic filtrate by the acid treatment even though the freshly isolated culture yielded the toxic filtrate used in the study.

Strain A211 had at various times been treated with various acids, including HCl, lactic and butyric, but none of the sluggish principle could be isolated.

Strains 101, A211, FBB were transferred without sporulating until the cultures failed to head and had become slimy but filtration failed to yield a toxic filtrate.

TABLE I

REACTION OF THE BUTYL STRAINS TO THE SLUGGISH PRINCIPLE

Excellent Head - EH; Good Head - GH; Fair Head - FH

Poor Head - PH; No Head - NH

Culture	Control Head 24 hrs.	Filtrate Head 24 hrs.	Control Acidity 70 hrs.	Filtrate Acidity 70 hrs.	Filtrate cc. N/10 acid per 10 cc. mash	
(1) <u>Cl. aceto-</u> <u>nigenum</u>	GH	NH	4.1	3.5	3.9	
(2) <u>Cl. buty-</u> <u>ricum</u>	EH	NH	2.9	5.7	4.7	
(3) W36	EH	NH PH	3.3	3.1	3.6	
Rettger's Cultures						
(4) SO	EH	NH	3.5	3.7	5.6	
(5) R	EH	PH PH	3.1	4.3	4.9	
(6) I	FH	NH	4.0	3.4	4.2	
(7) B	EH	NH	3.5	3.2	5.0	
Fernbach's Cultures						
(8) 12A	GH	NH PH	3.8	3.0	7.0	
(9) 12B	GH	PH	4.9	3.9	7.6	
(10) FBB	EH	EH	3.1	3.7	4.9	
(11) F1	GH	NH	4.1	4.7	5.5	
(12) 101	EH	NH	4.5	4.1	5.3	
(13) A211	EH	PH	3.5	3.2	4.7	
(14) MS	EH	NH	3.0	4.7	4.2	



The ninth and tenth subculture of A211 in tubes failed to head, but the eleventh subculture in 300 cc. of 5% mash in a 500 cc. Erlenmeyer flask produced an excellent head and then the twelfth subculture in tubes produced a good head.

Fermentation in the presence of excess  $\text{CaCO}_3$  using strains 101 and FBB failed to yield toxic filtrates even though the fermentation appearance was that of a sluggish culture and the 101 culture yielded volatile acid which accounted for nearly 40% of the cern. The yield of acetic acid was 13.7% and of butyric acid was 26.2% in 70 hours.

### Filtration Studies

Three types of bacterial filters were used in these experiments. The first type, the Berkefeld filter candle, was not extensively studied as regard the effect of pH on the filtration of the sluggish principle but by trial and error it was found that good results were obtained by filtering in a pH range from 3.8 to 4.0 while certain values on either side were less satisfactory.

The second type of filter used was the Jenkins filter but for this work there were two decided disadvantages. The filters employ metal in their construction and solutions filtered at low pH values were blue due to the copper dissolved from the filter. The filtering surface was small and the porosity of the filters used in this laboratory was low, making filtration very tedious.

The third type of filter used included the Chamberlain filter cylinders which were practically ideal for this work. These filters could be obtained having various porosities, and they could be set up in 500 cc. or 1 L. suction flasks so that no metal parts were exposed. A wide range of pH values could be studied without noticeably affecting the filters.

The method of filtering was to adjust the mash to the desired pH value and filter, first through ordinary filter paper, and then through the filter cylinder into a sterile suction flask. When the mash was filtered at low pH values a few grams of  $\text{CaCO}_3$  were placed in the flask before sterilizing. An automatic setup for filtration is shown in Fig. 1. The filtrate was transferred aseptically through the delivery tube of the suction flask into sterile tubes or flasks. The tube for admitting air during the drainage of the flask makes it possible to transfer the filtrate smoothly without air being sucked in at the delivery tube.

The porosities of the filters used were marked L7, L5, L3, L2. A series of filtrates prepared from the same mash at the same pH, varying the porosity as listed, were all potent, showing that the particle size for the sluggish principle was less than the smallest pore size. Size L5 has been used throughout in the other experiments.

The effect of pH on filtration was studied in order to determine the most suitable pH for filtration of the sluggish principle. The results are listed in Tables II and III.

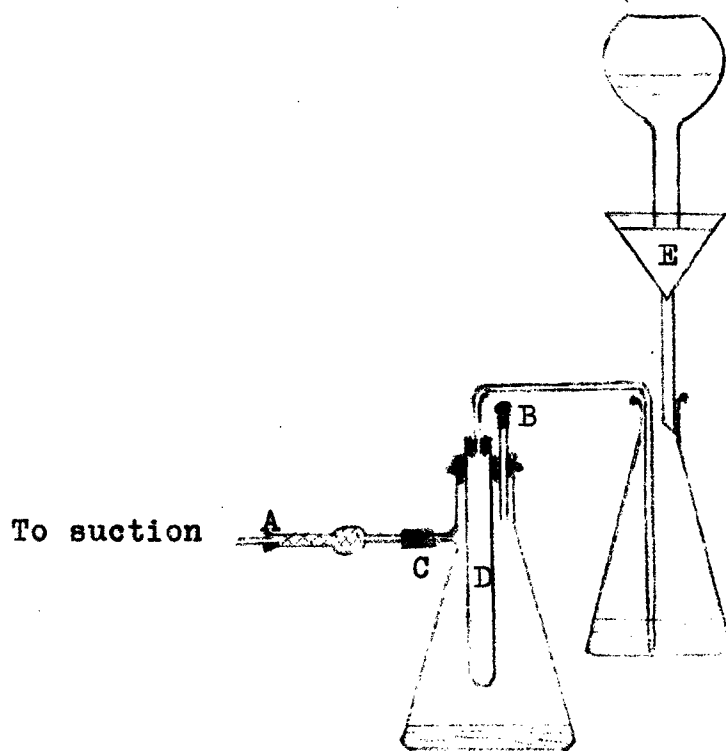


Fig. 1. A Filtration Set-up.

- A.  $\text{CaCl}_2$  tube filled with cotton.
- B. Glass tube plugged with cotton for admitting air during drainage of flask. This tube is closed with rubber tubing and a glass bead during filtration.
- C. Delivery tube for drainage of flask.
- D. Filter cylinder.
- E. Funnel and filter.

TABLE II

THE EFFECT OF PH ON THE FILTRATION OF THE  
SLUGGISH PRINCIPLE

<u>pH</u>	<u>: Reagent used for</u>	<u>: Head 40 hours</u>
	<u>: adjustment of pH</u>	<u>:</u>
3.1	Lactic acid	GH
4.0	Butyric acid	GH
5.1	NaOH	NH
6.0	NaOH	NH
7.0	NaOH	NH
7.1	NaOH	NH PH
8.2	NaOH	NH PH
9.0	NaOH	PH
10.1	NaOH	EH
Controls		EH

TABLE III

FILTRATION STUDIES

pH before filtering		pH after		Head at		Final acidity	
Glass : Quinhy-		: filtering		40 hours		66 hours	
Electrode: drone		:		:		: cc. N/10 per	
						: 10 cc. of mash	
4.05	: 4.22	: 4.28	:	NH	:	8.0	7.4
	: 4.35	: 4.40	:	GH	:	4.4	4.4
	: 4.40	: 4.19	:	FH PH	:	3.6	8.1
	: 4.54	: 4.46	:	NH	:	9.0	7.8
	: 4.68	: 4.50	:	NH	:	7.1	6.8
	: 4.80	: 4.81	:	NH	:	7.3	7.8
4.72	: 4.91	: 4.81	:	NH	:	8.7	8.5
	: 5.05	: 5.18	:	NH	:	8.6	7.7
	: 5.36	: 5.47	:	NH	:	9.5	9.2
6.00	: 5.65	: 5.67	:	NH	:	8.0	9.1
	: 6.08	: 5.58	:	NH	:	8.4	8.3
	: 6.18	: 6.10	:	NH	:	6.9	7.9
7.87	: 6.30	: 6.10	:	NH	:	8.4	7.8
	: 6.55	: 6.43	:	NH	:	8.3	8.4
	: 7.66	: 7.57	:	NH	:	9.0	9.0
9.07	: 8.76	: 7.65	:	NH	:	8.9	8.6
: Controls		:	-	:	EH	:	4.1 4.3

The preliminary tests listed in Table II indicated that there were wide limits of H-ion concentration for the filtration of the sluggish principle, ranging between pH 5.0 and pH 8.0.

The data in Table III confirm the conclusion drawn from the preliminary experiment. The filtration may appear poorer at extremely high or low pH values only because such concentrations may be detrimental to the sluggish principle.

A large number of filtrations were performed because it might have been possible to obtain some data which would have given information concerning the iso-electric points of the sluggish principle and of the filters. For example if the filter had possessed an iso-electric point at a pH of 4.0 and the sluggish principle had an iso-electric point at pH of 4.5 then a filtration at a pH of 4.25 would probably fail to yield any of the filterable sluggish principle since the filter and the material would be oppositely charged and the attraction of opposite charges would prevent a large portion of the filterable material from passing the filter. A filtration conducted at a pH outside of the values 4.0 and 4.5 would find like charges on the filter and the filterable material and filtration would proceed without

obstruction. It so happens that the filtration of the sluggish principle at pH values of 4.35 and 4.40 was apparently poorer than at pH values on either side. The graphic representation of the filtrations performed at various pH values is given in Fig. 2. The final acidity of the test fermentation has been used as a measure of the virulence of the filtrate and has been plotted against the pH of filtration.

Since the filtrates were to be stored, the pH value after filtration was determined, because the pH before and after filtration might not be the same. If the filter adsorbed H-ions more regularly than OH- ions the filtrate would become less acid while the filtrate would become more alkaline if the converse were true. There are noticeable differences in pH before and after filtration and the change is not altogether irregular. As shown in Fig. 3 there is a periodic change depending on the pH of filtration.

The pH values were obtained by means of a Leeds and Northrup outfit designed for use with a quinhydrone electrode. A saturated calomel half-cell was used as the opposite electrode. A few values were checked with the glass electrode. The agreement listed in Table III is fair considering that the medium is a rather complex mixture.



Final acidity  
cc. 0.1 N acid  
per 10 cc.

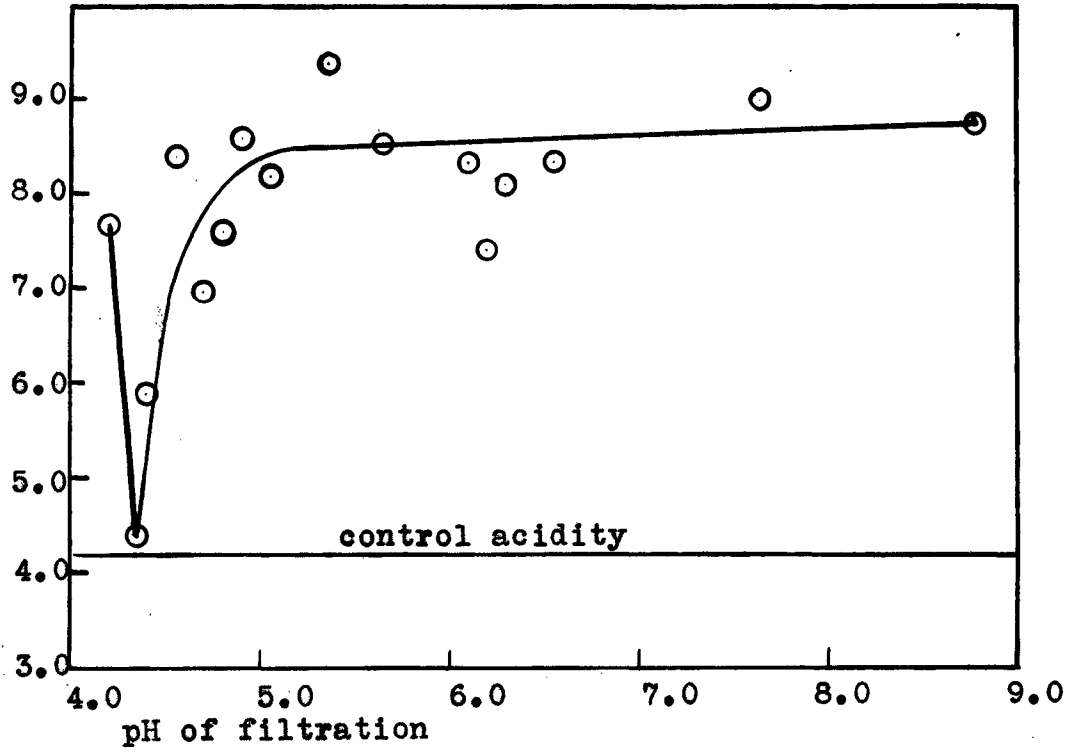


Fig. 2. Effect of pH on Filtration

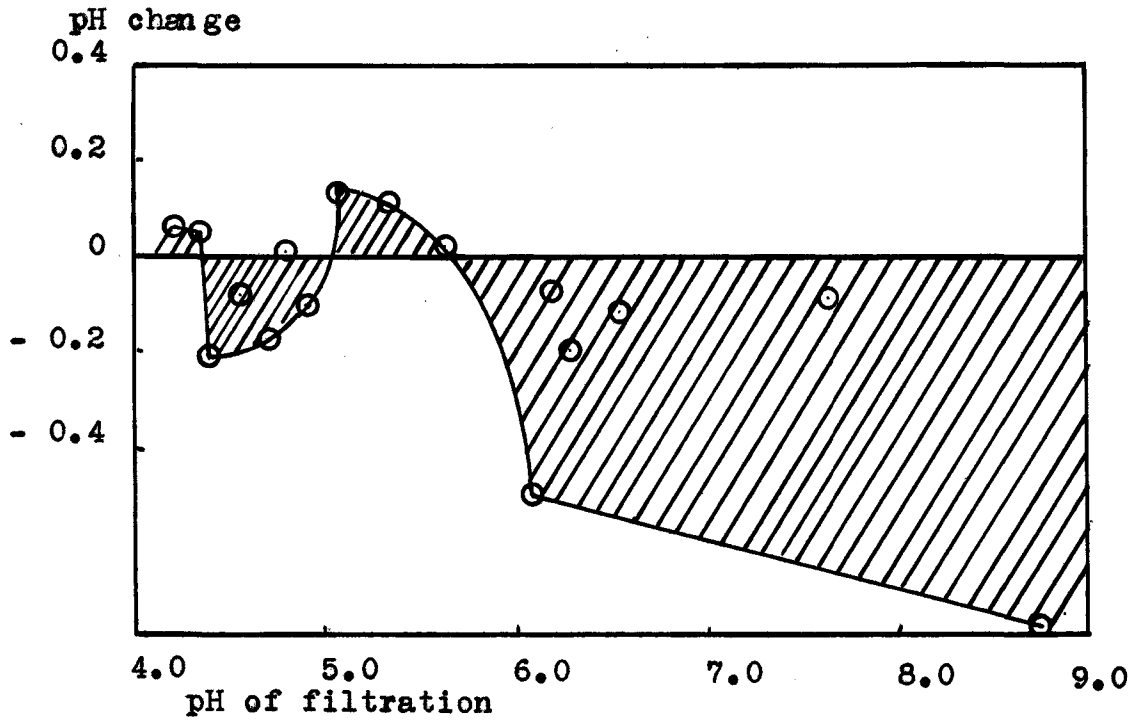


Fig. 3. Change in pH of Fermented Mash  
Due to Filtration

Immunization of Cultures Toward the Action of the

Sluggish Principle

Legg (27) describes a method by which butyl cultures may be immunized, as follows: "A sterile carbohydrate mash, consisting, for example of 6% corn mash, is prepared and is inoculated with a spore culture of butyl-acetonic bacilli. It is then 'heat-shocked' for three minutes at 100° C., after which there is added a small portion, one or more drops, of clear liquid obtained from a carbohydrate mash undergoing a sluggish butyl-acetonic fermentation, by filtering the mash first to remove all solids and second through a Berkefeld filter to remove bacteria. Fermentation is then allowed to proceed at a temperature of about 36° C. for four or more days -- i.e. until spores have developed. This spore culture is then used to inoculate fresh corn mash which is again 'heat-shocked' for about three minutes at 100° C., and treated with two drops of the filtrate as previously mentioned. The second fermentation is allowed to proceed as before and additional transfers with shocking and filtrate addition are made -- that is the process is repeated. At the end of about ten such treatments the culture thus obtained in spore form at the end of the last treatment will ordinarily be found to be immunized in respect to sluggish fermentation, and, if not the described treatment of the culture is continued until the immunity is attained."

Two strains, A211 and 101 were subjected to the Legg treatment but, after ten treatments, were still susceptible, as shown by observation of heads and final acidities and after eighteen treatments the cultures were very slimy and would scarcely grow at all. A modification of Legg's procedure was also tried. The cultures were started from spores in corn mash tubes, treated with two drops of filtrate, heat-shocked two minutes in boiling water and incubated at 37° C. The cultures were transferred every 24 hours and sporulated after the fifth or sixth transfer, and the most vigorously fermenting tubes were chosen each time for further study.

The above procedure was applied to six strains of butyl organisms, W3, A211, R, 101, F1, and MS and after six or seven cycles of the above treatment one out of the six strains, W3, apparently was quite resistant to the action of the sluggish principle. This new resistant culture was designated as W36 because the strain W3 had been treated six times. Of course it cannot be neglected that many workers have produced vigorous cultures by merely heat-shocking, transferring, and storing the spores on soil and Weyer and Rettger (52) have shown quite definitely that the acetone yield increased with successive heat treatments. These workers assume that the increased yield is

due to a selection of resistant spores which are capable of higher yields. Legg's claims (27) are similar concerning the increase in yields and it is possible that both investigators are accomplishing the same thing with their respective treatments, that is Weyer and Rettger may be eliminating small traces of the contaminating sluggish principle from their spores by successive pasteurizations as well as selecting resistant spores and Legg may be selecting more resistant spores during his procedure and both factors combined to give the increased yields observed. Weizmann (50) was probably accomplishing the same end by his 100 to 150 cycles of inoculation, "heat-shock" and incubation.

### The Properties of the Filtrate

The properties of the filtrate have been studied from two angles, one being the effect that the filtrate had upon the growth of the butyl organisms and the products formed by them while the other was concerned with the conditions which affected the toxicity of the filtrate.

The effect of the filtrate on the acidity curve is probably one of the most reliable criteria as to the presence of the sluggish principle, since the course of the acidity during the fermentation is a good measure of the general condition of the culture. There is one disadvantage to the acidity curve as a criterion, the stirring or shaking required to obtain uniform samples disturbs the culture somewhat and the action of the sluggish principle becomes less marked. Usually the final acidity of filtrate flasks being sampled runs lower than those remaining undisturbed. The experiments for this investigation have repeatedly been less conclusive in large flasks than in small flasks or tubes.

Typical acidity curves of strain 101 are shown in Fig. 4 compared with the acidity curve of Speakman's slow fermentation. The acidities were measured in 5% corn mash in 2 L.Erlenmeyer flasks containing 1700 cc. of the medium, 5 cc. of the toxic filtrate and 50 cc.

Acidity  
cc. 0.1 N acid  
per 10 cc.

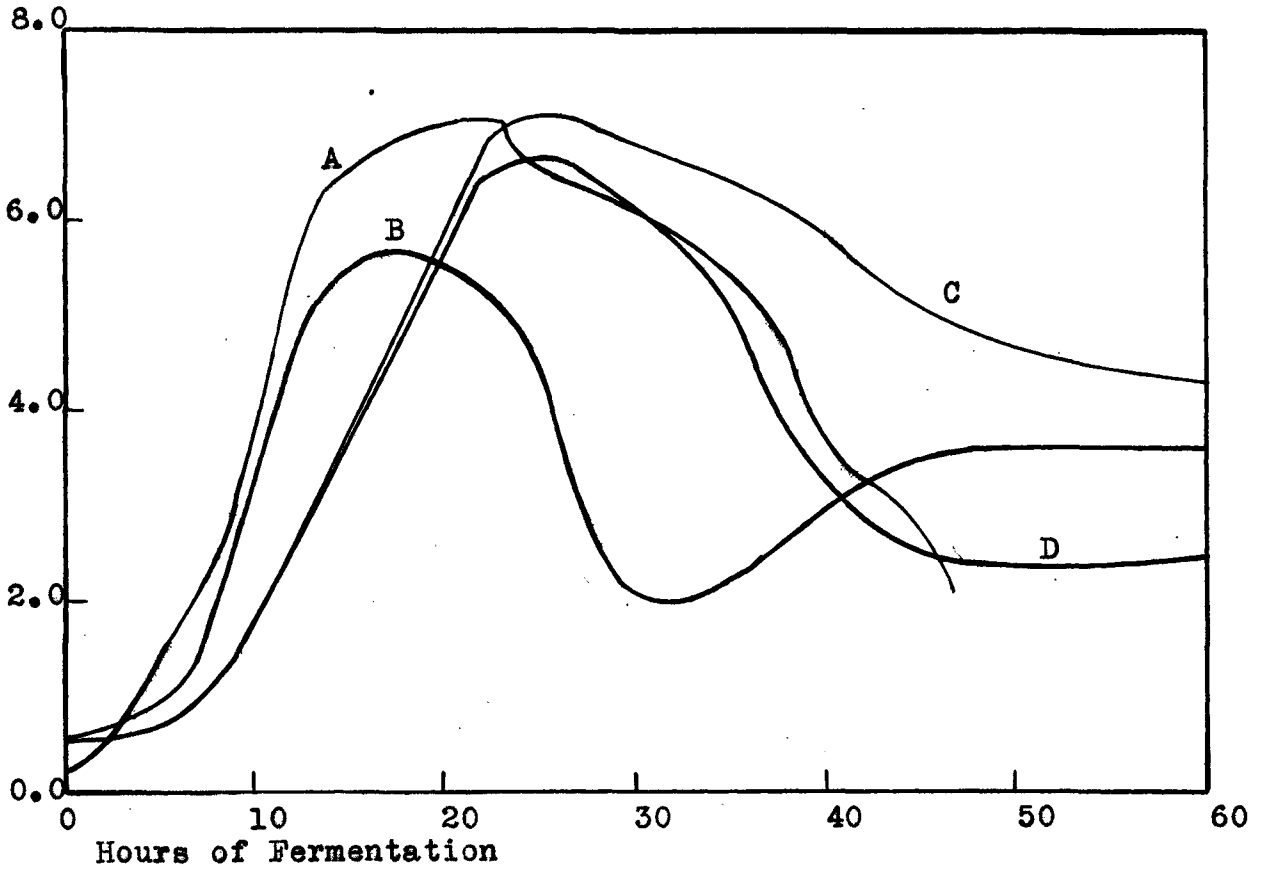


Fig. 4. Acidity Curves

A. Speakman's slow fermentation.

B. Normal fermentation, Strain 101, 5% corn mash, 37° C.

C. and D. Sluggish fermentations, Strain 101, 5% corn  
mash. 37° C.

of 24 hour inoculum. The samples were removed aseptically with wide tip sterile pipettes. A 10 cc. portion of each sample was diluted, heated just to boiling and cooled quickly to avoid loss of the volatile acid and titrated with 0.1 N NaOH to the phenolphthalein endpoint. Throughout this work the term, acidity, refers to the cubic centimeters of 0.1 N NaOH required to give the phenolphthalein endpoint in 10 cc. of mash.

Examination of the acidity curves will make it clear that occasionally in fermentations only mildly affected by the sluggish principle, the final acidity would fail to indicate the presence of any toxic material since the final acidity of the normal curve is between the final acidities of the filtrate flasks. On the basis of the final acidities only one of the two filtrate flasks would have appeared even slightly sluggish but the acidity curve shows both filtrate flasks were definitely slower than the control and the appearance of the heads also indicated sluggishness. The control flasks headed in 20 - 24 hours, while the sluggish flasks required 44 hours or more to produce heads, and these heads were not as compact nor were the liquids as clear as in the control flasks.

The method of comparing the products of a sluggish and a normal fermentation was merely to set up a carbon balance for each type and make a comparison.



This method was tried several times but frequently it has been observed that in 1 L. and 2 L. flasks a toxic filtrate would not produce a highly sluggish fermentation even though the same toxic filtrate would produce final acidities of 5.0 and 9.0 in tubes and small flasks.

During the first part of this investigation several attempts were made to collect all of the final products of a fermentation from a 1L. or 2 L. flask but in each case the flask would finally head, the acidity would decrease and nearly a normal yield of solvents was produced. Finally it seemed that a highly sluggish fermentation in these larger flasks inoculated with filtrate would be an exceptional rather than a usual occurrence. In tables IV and V are listed the carbon balances of both types, the normal fermentation produced by strain 101 and the fermentation of strain 101 contaminated with a toxic filtrate, but it must be repeated that the sluggish fermentation represented by the carbon balance listed is not an example of a highly sluggish fermentation.

The preparation of complete fermentation balances required a quantitative analysis of a complex mixture both before and after fermentation.

The corn meal was analysed for moisture and carbohydrate before the medium was prepared. The moisture determination consisted of drying the corn meal at 103<sup>o</sup> C. to 105<sup>o</sup> C.

TABLE IV

A FERMENTATION BALANCE FOR A NORMAL CULTURE

STRAIN 101

Mash 4.76 g. dry corn per 100 cc.

	: Grams :	Carbon :	Mols :	H :	-H :	+H :
	:	Grams :	:	value :	:	:
Starch(Initial)	: 61.00 :	27.10 :	.377 :	0 :		
Starch(Final)	: <u>4.26</u> :	<u>1.89</u> :	<u>.027</u> :	0 :		
Starch(Fermented)	56.74 :	25.21 :	.350 :	0 :		
H <sub>2</sub>	: 1.122:	- :	.561 :	2 :		: 1.122
CO <sub>2</sub>	: 35.50 :	9.66 :	.808 :	-4 :	-3.232	
Butanol	: 11.55 :	7.50 :	.156 :	8 :		
Acetone	: 6.24 :	3.87 :	.107 :	4 :		: .428
Ethanol	: 2.00 :	1.04 :	.044 :	4 :		: .176
Acetylmethyl carbinol	.45:	.25 :	.0051:	4 :		: .020
Butyric acid	: 1.31 :	.71 :	.015 :	4 :		: .060
Acetic acid	: 2.54 :	1.02 :	.042 :	0 :		: -
Formic acid	: Trace :	- :	- :	-2 :		: -
		24.05 :			-3.232 :	3.054

Carbon accounted for 95.4%

Solvent ratio in grams 5.78 : 3.12 : 1.00

Mols CO<sub>2</sub> : Mols H<sub>2</sub> 59.0 : 41.0

Mols acetic acid : Mols butyric acid 2.8 : 1.0

TABLE V

A FERMENTATION BALANCE FOR STRAIN 101

CONTAMINATED WITH FILTRATE

	: Grams :	Carbon :	Mols :	H :	-H :	+H :
	:	Grams :	:	value :	:	:
Starch(Initial)	61.00:	27.10 :	.377 :	0 :		
Starch(Final)	<u>5.50:</u>	<u>2.46 :</u>	<u>.034 :</u>	<u>0 :</u>		
Starch(fermented)	55.50:	24.64 :	.343 :	0 :		
H <sub>2</sub>	: 1.095	- :	.544 :	2 :		
CO <sub>2</sub>	: 35.45:	9.67 :	.805 :	-4 :	-3.220	
Butanol	: 11.51:	7.46 :	.155 :	8 :		1.240
Acetone	: 6.00:	3.72 :	.103 :	4 :		.412
Ethanol	: 1.96:	.97 :	.040 :	4 :		.160
Acetylmethyl carbinol	: .314:	.18 :	.0036:	4 :		.014
Butyric acid	: 1.61 :	.88 :	.018 :	4 :		.072
Acetic acid	: 2.59 :	1.03 :	.043 :	0 :		-
Formic acid	: Trace :	- :	:	-2 :		-
		<u>23.91 :</u>			<u>-3.330:</u>	<u>2.986</u>

Carbon accounted for 96.9%

Solvent ratio in grams 6.19 : 3.16 : 1

Mols CO<sub>2</sub> : Mols H<sub>2</sub> 59.7 : 40.3

Mols acetic acid : Mols butyric acid 2.4 : 1.0

to constant weight and determining the loss in weight. The three or four hours required for drying did not discolor the corn meal. The carbohydrate estimation was performed by acid hydrolysis of the starch (55) and the subsequent determination of the reducing sugars by the method of Shaffer and Hartmann (39). The total amount of carbohydrate was calculated as starch. The particular sample of corn meal used for these fermentations contained 11.38% moisture and 65.2% carbohydrate, calculated as starch. The media consisted of 93.5 g. of corn meal in a volume of 1740 and 1760 cc. after sterilization, or 4.76 g. and 4.71 g. of dry corn per 100 cc. of mash.

The complex fermentation mixture was analysed for solvents, including n-butanol, acetone and ethanol, volatile acids, acetylmethyl carbinol, and the residual carbohydrate was determined as described above. The non-volatile acids were extracted with ether and titrated with 0.1 N NaOH. On the basis of the work of Stiles, Peterson, and Fred (46) the non-volatile acids are probably not dissimilation products of the carbohydrates but come from the protein material, and in either case were only 5% of the total acidity.

The method used in the solvent estimation was to measure 300 cc. of the beer into a distilling apparatus

with a slight excess of  $\text{CaCO}_3$  or an equivalent amount of  $\text{NaOH}$  solution and distill 100 cc. into a volumetric flask. The receiver was kept cool in a trough of running water. The specific gravity of the distillate was determined with a 25 cc. pycnometer at  $25^\circ$ . From data on the specific gravities of the known solutions of solvent mixtures in a ratio of 6 g. n-butanol, 3 g. acetone and 1 g. ethanol, the following equation for the calculation of the total solvents (T) was derived,

$$T = \frac{1.00000 - \text{Sp.G. } \frac{25}{25}}{0.00152}$$

The distillate was diluted 10 to 100 for the Messinger method for determining acetone, as outlined by Goodwin (21). This method was checked on prepared solutions containing the same amounts of n-butanol, acetone, and ethanol as found in the fermentation distillates and the error due to the presence of ethanol was not over one part in 100. The alcohols were determined by measuring their reducing power against  $\text{K}_2\text{Cr}_2\text{O}_7$  and  $\text{H}_2\text{SO}_4$ . The conditions were maintained so that the oxidation of ethanol to acetic acid as measured by the  $\text{K}_2\text{Cr}_2\text{O}_7$  used was practically quantitative. These conditions caused the oxidation of n-butanol to use more  $\text{K}_2\text{Cr}_2\text{O}_7$  than that required by the theoretical conversion of n-butanol to n-butyric acid. In order to

calculate the concentration of both alcohols, it was necessary to titrate the original distillate and record the amount of  $K_2Cr_2O_7$  used ( $M_1$ ) and then extract with a double volume of  $CCl_4$  at 25 °C. and titrate the alcohols remaining in the aqueous layer. The  $K_2Cr_2O_7$  used ( $M_2$ ) for the second titration was recorded and the concentrations (expressed as g. per 100 cc.) of the alcohols in the distillate calculated from the following empirical equations, derived from data covering a large variety of solutions of known concentration.

$$\text{Equation 3 } BuOH = 0.25 (M_1 - M_2) - 0.01 M_1 - 0.05 \text{ Acetone}$$

$$\text{Equation 4 } EtOH = 0.118 M_1 - 0.30 BuOH - 0.04 \text{ Acetone}$$

$$\text{Equation 5 } EtOH = 0.22M_2 - 0.09 M_1 - 0.01 \text{ Acetone}$$

$$\text{Equation 6 } EtOH = 0.124 M_2 - 0.35 BuOH - 0.03 \text{ Acetone}$$

Corrections<sup>#</sup> must be applied to the values obtained from all of the equations, excepting Equation 4, due to the fact that  $M_2$  is not a linear function of the concentration.

The values for the ethanol concentration were calculated using three different equations and the average values did not differ by more than three parts per hundred even for the small amounts found in these fermentation distillates, which contained relatively large amounts of

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<sup>#</sup> This method has been developed by several workers in this laboratory and details will be published later.

n-butanol. The accuracy was also checked by comparing the total solvent concentration found using specific gravity with the sum of the calculated concentrations of n-butanol, acetone, and ethanol.

	Distillate from Normal Flask g. per 100 cc.	:	Distillate from Filtrate Flask g. per 100 cc.
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Total solvents by specific gravity	3.50	:	3.30
Sum of n-butanol, acetone, and ethanol	3.46	:	3.34

The volatile acids were distilled in an apparatus which provided for the addition of distilled water during the distillation. The residue containing the Na salts that remained after the distillation of the solvents was acidified with 10 cc. of 5 N sulfuric acid and distilled until about 100 - 150 cc. of liquid remained in the flask. Then distilled water was added to keep the volume constant while the distillation continued, producing in all 500 cc. of distillate. This type of separation for volatile acids is not entirely quantitative but since more than 96% of the total acidity was obtained in the distillate the recovery of volatile acid must have been quite complete.

The chemical method of Fyfe (15) was used for estimating the butyric acid in the volatile acid mixture. The method is based on the reducing power of butyric

In a  $K_2Cr_2O_7$  and  $H_2SO_4$  solution under specified conditions. There is one precaution that must be observed and that is to boil the solution of the volatile acids, containing a slight excess of  $NaOH$ , nearly to dryness three or four times to eliminate the high boiling materials which are carried over in small amounts during the steam distillation. The acetic acid was obtained by difference. The distillate was analysed for formic acid by refluxing 100 cc. with  $HgCl_2$  solution but only questionable traces of  $Hg_2Cl_2$  were precipitated.

The fermented mash was treated to estimate the amount of acetylmethyl carbinol as directed by Wilson, Peterson and Fred (54). The acetylmethyl carbinol was oxidized with  $PbCl_2$  to diacetyl (B.P.  $88^\circ C.$ ) which readily distilled over and was precipitated by nickel and hydroxylamine as the nickel salt of dimethyl glyoxime. These authors and Donker (4) all believe that the method gives results which are too low. In connection with this determination the solvent distillates were examined for the presence of acetylmethyl carbinol since the acetylmethyl carbinol in appreciable amounts would use part of the  $K_2Cr_2O_7$  in the determination of the alcohols. The boiling point of acetylmethyl carbinol is  $140^\circ - 144^\circ C.$  and this explains why only traces of the compound were found in the solvent distillates. The amount would probably be entirely negligible



in the  $K_2Cr_2O_7$  used.

The fermentation gases were collected in a 20 L. bottle over saturated NaCl solution. The volume was measured by removing the gas with a calibrated 5 L. gas container which was fitted with a leveling bulb. The volume could be read within 2 - 3 cc. A 100 cc. gas burette was used to adjust the volume of gas in the large gas bulb to the mark. It was found that the gas could be also read directly from marks on the side of the 20 L. bottle. If corrections were made for the difference in level of the salt brine (Sp. G.  $1.20 \frac{36}{25}$ ) in and outside of the bottle the reading obtained directly from the bottle agreed with the more precise readings obtained with calibrated gas bulb within 2 to 4 parts per thousand. In both cases the volumes were corrected for the vapor pressure of the saturated NaCl solutions at their respective temperatures. These values were obtained from the tables published by Leopold and Johnson (28).

The gas mixture was analysed with a Williams apparatus. The values for the carbon dioxide absorbed by KOH on duplicate determinations checked within two parts per thousand and the hydrogen percentage could be made to agree within two or three parts per thousand by choosing the proper amount for explosion. A moderate excess of oxygen was used to explode the hydrogen. Frequently the air in the gases was estimated by absorbing the oxygen

in alkaline pyrogallate solution and multiplying the oxygen percentage by five. The fermentation flask was swept with three or four liters of CO<sub>2</sub> free air after the gasing had ceased. The CO<sub>2</sub> dissolved in the mash was estimated from the difference between the boiled and unboiled acidity but it was only a very small percentage of the total carbon dioxide.

The final carbon and oxidation-reduction balances similar to those of Johnson, Peterson and Fred (24) except that H values were recorded instead of H<sub>2</sub> values also serve to check the accuracy of the analytical procedures in general although there are undoubtedly compensating errors. The carbon balance of the normal fermentation agrees fairly well with that of Donker (4) for the Weizmann bacteria in 4% corn mash. See Table VI. The values for acetylmethyl carbinol are farthest apart. It is interesting to note that even though there is not very good agreement between the n-butanol values and only fair agreement between the acetone values, that if the total solvents are compared the check is very surprising. It seems possible that part of the ethanol may be included in Donker's value for n-butanol.

Since the larger flasks did not produce highly sluggish fermentations, smaller flasks were inoculated with a toxic filtrate and a susceptible culture, allowed to ferment, analysed and compared with similar analyses on normal cultures.

TABLE VI

DONKER'S VALUES FROM 4% CORN MASH  
COMPARED WITH STRAIN 101 IN 4.76% CORN MASH

	: Donker's	: Converted to	: Strain 101
	:	: same am't of	: 4.76 g. dry corn
	:	: starch as	: per 100 cc.
	:	: fermented by	:
	:	: Strain 101	:
Added Glucose	: 53.9	:	:
Unfermented "	: 2.2	:	:
Fermented "	: 51.7	: 56.7 g. starch:	56.7 g. starch
	: (46.5)	:	fermented
CO <sub>2</sub>	: 26.5	: 34.8	: 35.5
H <sub>2</sub>	: .887	: 1.082	: 1.122
Formic acid	: absent	: -	: -
Acetic acid	: 1.76	: 2.14	: 2.54
Butyric acid	: 1.0	: 1.22	: 1.31
Lactic acid	: Traces	: -	: -
n-Butanol	: 11.7	: 14.28	: 11.55
Acetone	: 4.56	: 5.56	: 6.24
Ethanol	: not deter- mined	: -	: <u>2.00</u>
Total Solvents		19.84	19.79
Acetylmethyl carbinol	1.23	: 1.5	: .45
2 - 3 Butylene glycol	: absent	: -	: not determined

An experiment was performed with 200 cc. of 5% corn mash in 300 cc. flasks inoculated with two drops of filtrate and 5.0 cc. of a 24 hr. 101 culture. Forty fermentations were run including six control flasks and thirty-four filtrate flasks. The final acidities of the filtrate flasks were fairly high and the fermentations were very sluggish, appearing much like the sluggish fermentations in tubes both as to acidity and heads. Consequently six of the filtrate flasks were chosen to be analysed and compared with the control flasks for solvents, volatile acids and residual carbohydrate.

The acidities were calculated from the amount of 1.0 N NaOH solution required to neutralize the 200 cc. of mash before distilling the solvents and therefore the mash was not boiled before titrating. The unboiled acidities may be 1.0 - 1.5 cc. higher than the boiled acidities especially in the controls due to the large amount of CO<sub>2</sub> trapped by the thick slimy head. However the values are useful for comparative purposes.

Fifty cubic centimeters of distillate were collected from the 200 cc. of mash and analysed for total solvents by the specific gravity method.

The volatile acids were distilled as previously described but the ratio of butyric acid to acetic acid was determined by Virtanen's distillation method (48).

These determinations were made before the method of Fyleman was introduced into this laboratory.

The residual carbohydrates were hydrolysed by 2%  $H_2SO_4$  during the distillation of the volatile acids and may not represent absolute values but are sufficiently accurate for comparison.

The results tabulated in Table VII represent the outstanding differences to be expected between the normal fermentation and the sluggish fermentation which gives a lower solvent yield and higher volatile acid content, with relatively incomplete utilization of the carbohydrate. It is rather striking that the ratio of butyric acid to acetic acid is higher for the sluggish fermentation and is fairly close to the ratio found at the acidity peak by Stiles, Peterson, and Fred (47).

The gas curves from a sluggish fermentation of 5.00% corn mash are shown in Fig. 5. This fermentation was typical of those observed in one liter flasks inoculated with a toxic filtrate and a susceptible culture. The inoculation ratio was 2.5% of a vigorous twenty-four hour culture of strain A211. The fermentation produced a good head after 44 hours while normal fermentations produced heads in 20 - 24 hours. The maximum rate of gas production occurred at the forty-fifth hour but the

COMPARISON OF THE PRODUCTS FORMED IN A NORMAL  
FERMENTATION AND A SUGGISH FERMENTATION

TABLE VII

Plank No.:	Total	Acidity	Butyric	Acetic	Residual	Solvents:	acid	acid	carbohydrate	starch	N	P	N	P	N	P	N	P
1	4.54:1.07:31	4.7	10.3:	0.51:	254:	104:	255:	197	1.60	:	:	:	:	:	:	:	:	:
2	4.54:1.14:36	5.6	9.0:	0.72:	218:	100:	238:	-	1.55	:	:	:	:	:	:	:	:	:
3	7.66:1.10:26	4.9	10.3:	0.89:	258:	098:	248:	1.66	1.57	:	:	:	:	:	:	:	:	:
4	7.66:1.25:25	4.8	10.4:	0.79:	286:	098:	231:	1.77	1.80	:	:	:	:	:	:	:	:	:
5	4.91:1.15:28	5.2	10.1:	0.78:	254:	102:	261:	1.58	1.77	:	:	:	:	:	:	:	:	:
6	4.91:1.14:28	4.9	9.8:	0.69:	276:	105:	238:	1.58	1.90	:	:	:	:	:	:	:	:	:

Unboiled acidity expressed as 0.1 N per 10 cc. mash.

Other concentrations G. per 100 cc. mash.

Mols acetic acid : Mols butyric acid = 2.0 : 1.0 Normal ratio  
 Acetic acid : Butyric acid = 1.3 : 1.0 Suggish ratio

Rate, - 51 -  
cc. per hour  
per liter of 5% corn mash

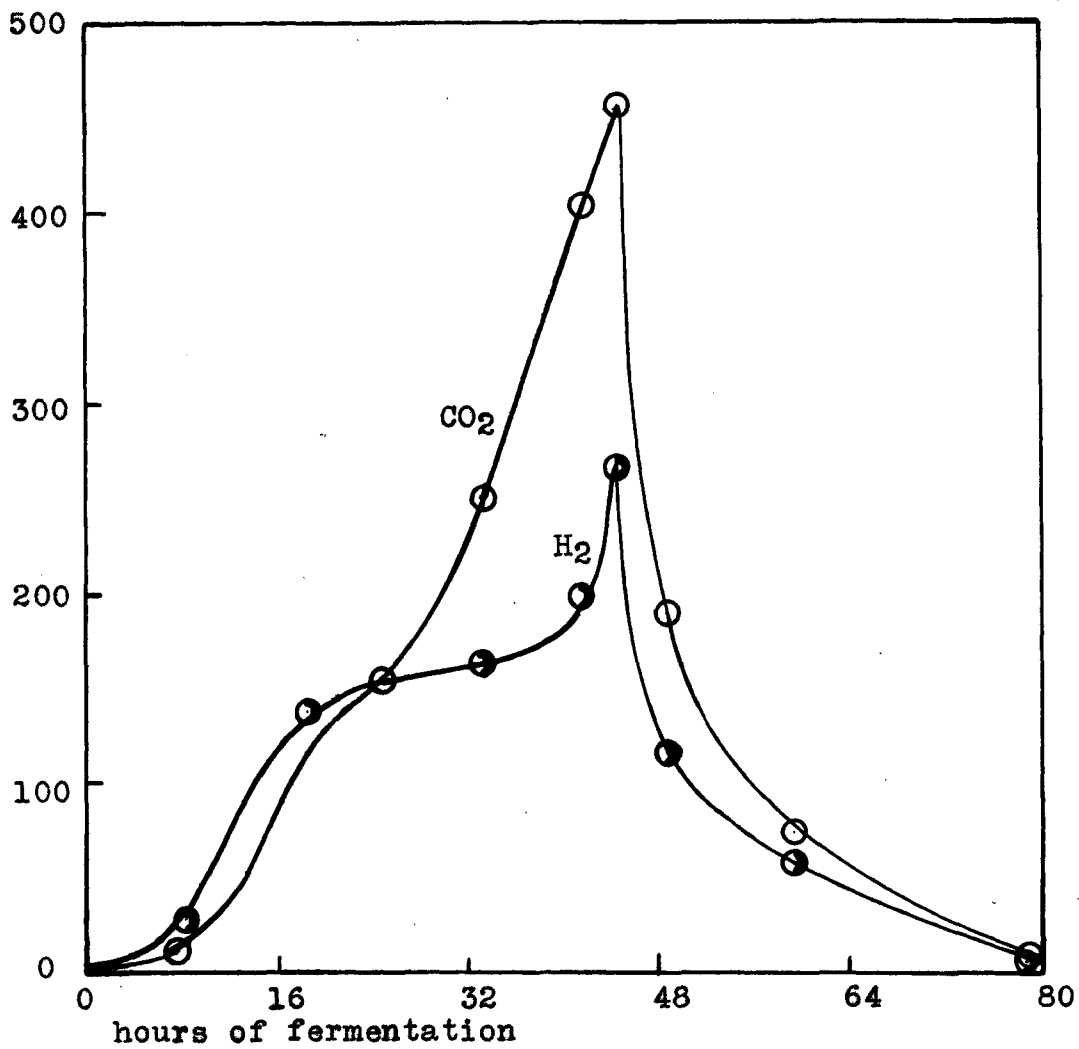


Fig. 5. Gas Curves of a Sluggish  
Fermentation

maximum for a normal fermentation occurred at the thirtieth hour. The solvent yield and final acidity after 80 hours of fermentation was practically normal.

The irregularity of the sluggish principle in large and small flasks was attributed to a variation in the oxygen relationship. If the organisms were more adaptable to traces of oxygen than the sluggish principle, then the tubes and small flasks which were easily and quickly swept free of air by fermentation gases would have become more sluggish than the large flasks. There was some qualitative evidence for believing that the sluggish principle was more susceptible to traces of oxygen than the butyl organisms. It was known, during epidemics of sluggishness in the fermentation industry, that aeration of the fermenter was sometimes helpful in obtaining a better solvent yield, and as was previously mentioned, flasks containing a toxic filtrate usually have higher acidities if they are not disturbed than if they are shaken or stirred occasionally.

It was hoped that some semi-quantitative information might be obtained concerning the effect of the oxygen tension on the action of the sluggish principle by varying the surface of a given amount of mash. A surface coefficient was calculated as follows,

$$\frac{\text{the area of the surface in cm}^2}{\text{the volume of the mash in cm}^3} = \text{cm}^{-1}$$



The flasks which were used in an anaerobic jar were considered as having a surface coefficient of zero. The results are listed in Tables VIII and IX. The acidity and total solvent values in Table VIII are in line with the hypothesis advanced but the solvent ratios were not complete due to the fact that the alcohols were calculated as n-butanol using Zeiss refractometer readings (R) and  $(M_1)^{\#}$  with the acetone values. The values in Table IX were obtained from  $(M_1)$   $(M_2)^{\#}$  values and are based on more reliable data but the fermentations were only mildly sluggish and the solvent yields were fully equal to the normal yield. The data represent the difference observed in normal fermentations as the surface coefficient is varied from 0 to 0.6 cm.<sup>-1</sup>. Apparently a vigorously fermenting butyl culture in corn mash maintains its own anaerobic conditions in spite of a moderate change in the surface coefficient. The filtrate flasks listed indicate higher n-butanol ratios and lower ethanol ratios.

The curves in Fig. 6 represent the results of an experiment in which varying amounts of filtrate were added to 300 cc. of 5% corn mash and inoculated with a normal 24 hr. susceptible culture. The solvents were distilled off after the mash had been neutralized with NaOH and the refractive number of the distillate at 25° C.

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# For explanation of  $(M_1)$  and  $(M_2)$  see page 42.

TABLE VIII

VARIATION OF OXYGEN TENSION

Surface Coefficient:		Acidity :		Total Solvents :		Butanol :		Acetone	
N	F	N	F	N	F	N	F	N	F
0	0	2.4	5.3	1.44	.60	.93	.41	.51	.19
0	0				.65		.49		.16
.13	.12	3.3	3.6	1.30	.81	.76	.58	.54	.23
.13	.12			1.31	.81	.78	.55	.53	.25
.40	.40	3.1	3.7	1.31	.81	.84	.56	.47	.26
.40	.40			1.27	.79	.78	.54	.49	.25
.63	.63	2.9	3.9	1.30	.71	.77	.49	.53	.22
.63	.63			1.14	.69	.71	.48	.43	.21

Values were calculated using equation,

$$\text{BuOH} = .764 R - .1453 M_1 - 1.387A - 10.08,$$

derived from data on prepared solutions containing known amounts of butanol, ethanol, and acetone. R = Zeiss immersion refractometer reading 25°; M<sub>1</sub> = same as in equation 3 and 4.

A = Acetone concentration.

$$\text{Surface Coefficient} = \frac{\text{Area in cm.}^2}{\text{Volume in cm.}^3} = \text{cm}^{-1}$$

TABLE IX

VARIATION OF OXYGEN TENSION

Surface Coefficient: Cm <sup>-1</sup>	Acidity:		Total Solvents:		Butanol:		Acetone:		Ethanol:		
	N	F	N	F	N	F	N	F	N	F	
0	0	3.4	3.6	1.03	1.05	.65	.68	.29	.32	.09	.05
0	0	2.9	3.3	1.04	1.06	.58	.66	.34	.35	.12	.05
0	0	3.6	3.2	1.02	1.09	.65	.70	.29	.33	.08	.05
.14	.14	3.6	3.7	1.10	1.03	.71	.65	.33	.34	.07	.04
.14	.14	3.4	3.2	1.03	0.79	.65	.51	.29	.25	.08	.03
.14	.14	3.2	3.8	1.05	1.02	.66	.67	.30	.31	.09	.04
.25	.26	3.6	3.4	0.98	1.01	.64	.68	.26	.28	.08	.05
.25	.26	3.4	3.4	0.95	1.05	.61	.68	.27	.32	.08	.05
.25	.27	3.8	3.3	0.96	1.07	.62	.70	.27	.33	.07	.05
.80	.78	3.7	3.6	0.89	0.98	.59	.64	.25	.29	.06	.05
.80	.78	3.4	3.0	0.92	0.92	.60	.61	.26	.27	.07	.04
.80	.79	3.2	2.9	0.84	0.96	.52	.65	.25	.28	.07	.03

Values were calculated using M<sub>1</sub>, M<sub>2</sub> and Acetone and equations 3 and 5. Surface coefficient =

$$\frac{\text{Area in cm.}^2}{\text{Volume in cm.}^3} = \text{cm}^{-1}$$

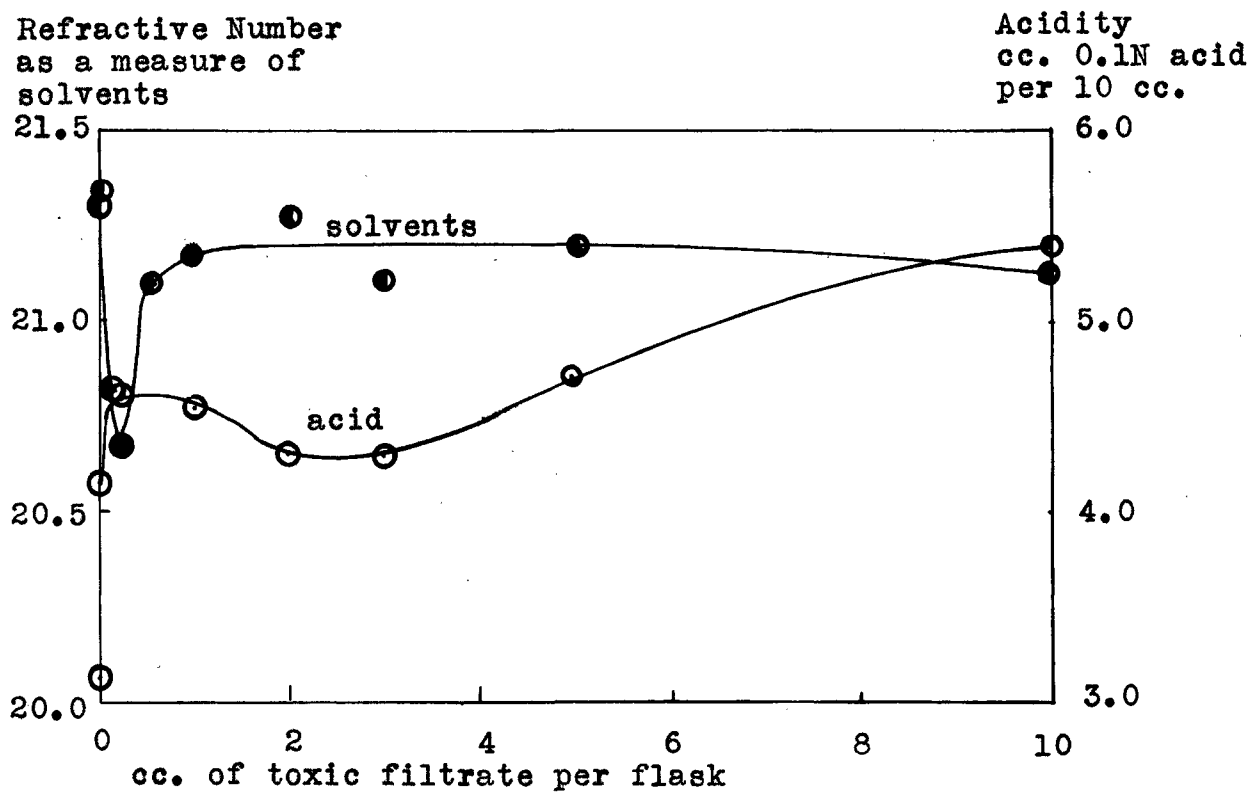


Fig. 6. Variation of Amount of Filtrate per Flask  
and the Effect on Solvent Yield and  
Final Acidity

taken as a measure of the solvents produced. The fermentations were not highly sluggish but there is enough difference to show that the presence of a small amount of the filtrate may be just as effective as a large amount.

The acid produced was in agreement with the solvent yields showing that the toxic material was catalytic or autogenic in nature rather than a supply of substance from which acid was produced.

The cell counts plotted in Fig. 7 were obtained by the Fries differential method of counting (14). The population of bacterial cells per cubic centimeter of 101 culture in 5% mash was nearly the same as reported by McCoy, Fred, Peterson and Hastings (31).

The methylene blue technic was to mix 20 cc. of the fermenting mash with 1 cc. of 0.5% methylene blue solution and incubate at 37°C. The methylene blue reduction values in Fig. 7 were obtained by incubating in an air bath maintained at 37°C. but the values in Fig. 8 were recorded after incubation in a water bath at 37°C. The disappearance of the blue color was chosen as the endpoint, but frequently slight greenish tinges remained at some stages. The surface remained blue after the main portion of the tube had become colorless. The determinations on duplicate flasks usually checked within one or two minutes except at the stages when periods of 30 minutes or more were required for

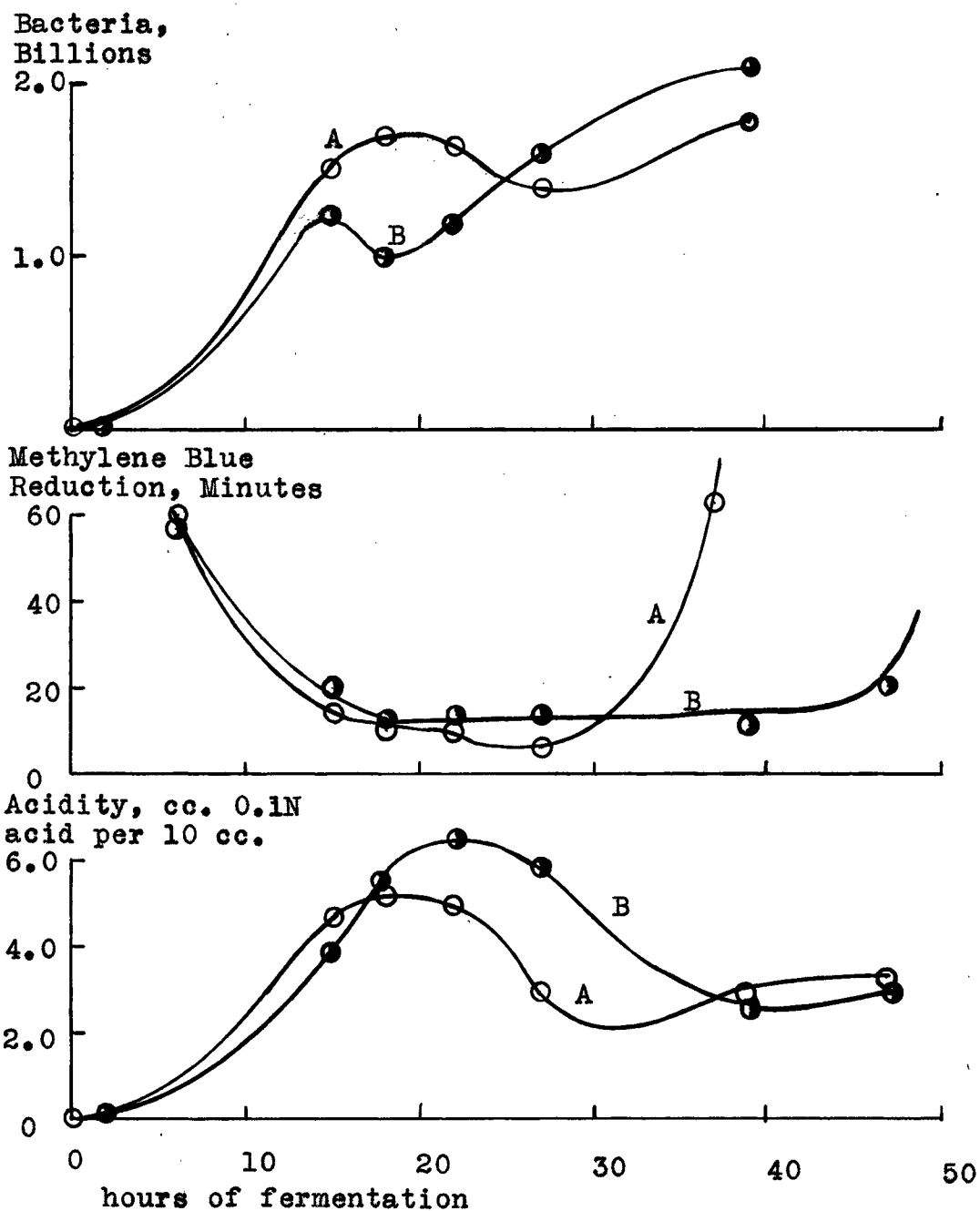


Fig. 7. Comparison of Normal Fermentations

A. Normal fermentation, Strain 101, 5% corn mash, 37°C.

B. Sluggish fermentation, Strain 101, 5% corn mash, 37°C.

reduction to take place.

The toxic material in the filtrate depressed the cell reproduction for a time as is shown by the curves in Fig. 7, but the same phenomenon occurred as that described above for large flasks. The culture appeared sluggish for 36 hours but then seemed to exert itself to overcome the effect of the sluggish principle and then the cell count exceeded that shown by the normal culture. The acidity curves were included to give a measure of the progress of the fermentation.

Fred and Peterson (36) have shown that their cell counts and the time required for methylene blue reduction were correlated and in this experiment the data on the reduction of methylene blue indicates that the sluggish fermentation was slower in starting but remained active for a longer period of time.

A study was made to determine the effect of the toxic filtrate on the hydrolytic processes of the butyl organism in corn mash. The results are represented graphically in Fig. 8. The reducing sugars were produced more rapidly in the normal fermentation than in the sluggish one. The Shaffer Hartmann method (39) of determining reducing sugars gives results that are too high because there are other substances besides glucose in the reaction

Methylene Blue  
Reduction  
Time in  
Minutes

- 60 -

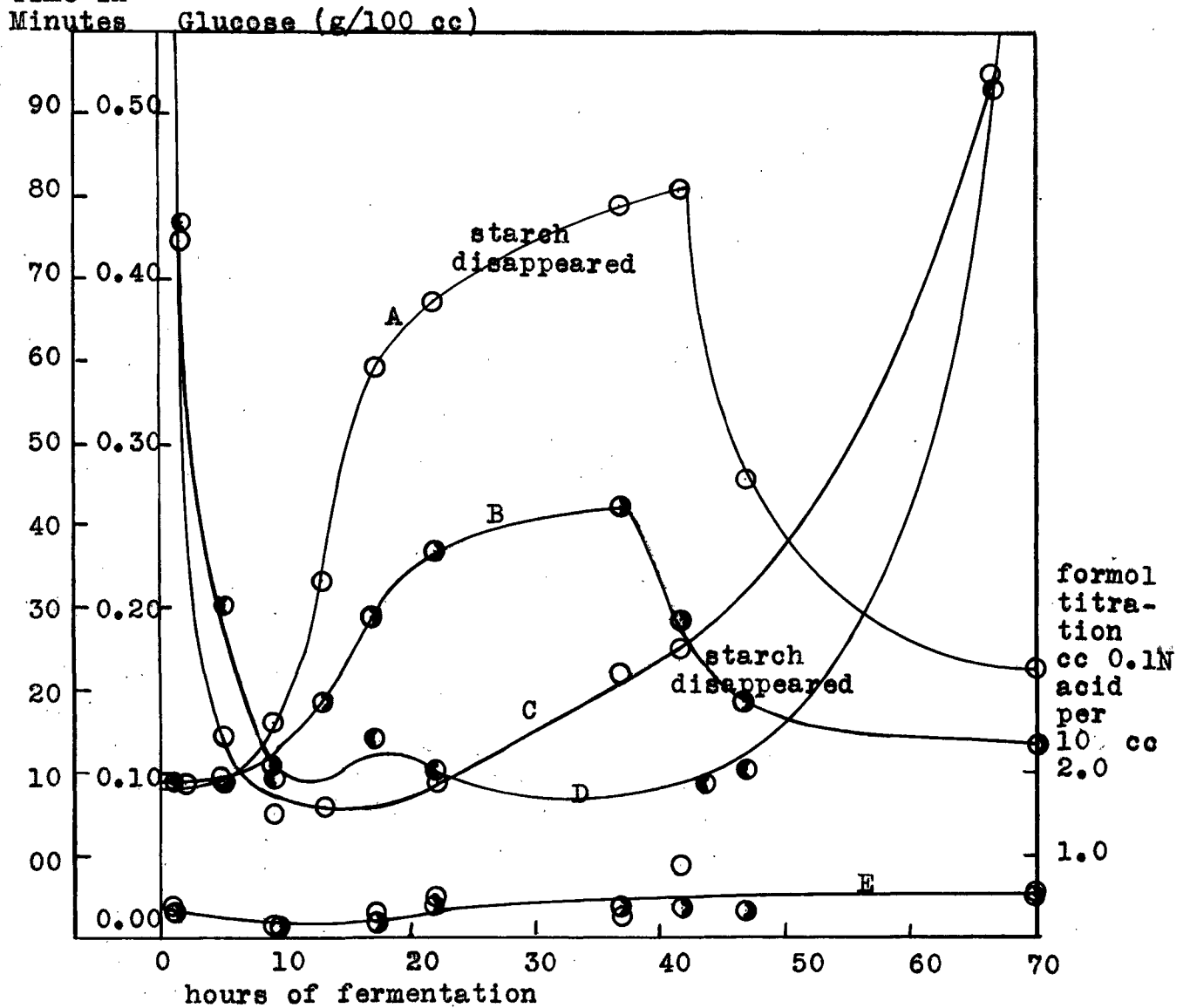


Fig. 8. A Comparison of Normal and Sluggish Fermentations.  
 A. Reducing sugars, calculated as glucose, in a normal fermentation Strain 101, 5% corn mash, 37°C.  
 B. Reducing sugars, calculated as glucose, in a sluggish fermentation, Strain 101, 5% corn mash, 37°C.  
 C. Methylene blue reduction in a normal fermentation.  
 D. Methylene blue reduction in a sluggish fermentation.  
 E. Formol titrations in normal and sluggish fermentations.



mixture which will reduce Fehling's solution, for example acetylmethyl carbinol and traces of aldehydes which might be present as intermediate products. The results were satisfactory at the beginning of the fermentation since the interfering substances were produced in the latter part of the fermentation and even then the results may be used for comparative purposes. It was evident there was a distinct lack of reducing substances in the sluggish fermentation when compared with the normal fermentation.

A formol-titration of Sorensen was used as a convenient check on the proteolytic difference between a normal fermentation and a fermentation in the presence of a toxic filtrate. The formol-titration in a normal fermentation even after 100 hrs. was less than 0.75 cc. of 0.1 N base for 10 cc. of the medium and no significant difference was observed between the normal and the sluggish fermentation. The amino-nitrogen calculated from the above formol titration on the assumption that there were the same number of carboxyl groups present as amino groups was 0.21% of the corn which is close to the value 0.24% reported by McCoy and others (31) using Van Slyke's method for determining the percentage of amino-nitrogen.

In order to obtain further information concerning the effect of the sluggish principle on the proteolytic activities of the butyl organisms, Levine and Carpenter's (29)

methods were applied in a study of the growth in gelatin. The media were made up as suggested by Levine and colleagues (29,30) except that both plain and glucose gelatin were tried. The compositions of the media are given below.

<u>Plain Gelatin</u>	<u>Glucose Gelatin</u>
20 g. gelatin (Bacto.)	20 g. gelatin (Bacto)
1 g. peptone (Bacto)	20 g. glucose
1000 cc. distilled water	1000 cc. distilled water
	1 g. peptone (Bacto)

The media were sterilized in 200 cc. portions for 15 min. at 15 pounds pressure in 300 cc. Erlenmeyer flasks. After inoculation, samples were removed at intervals aseptically and viscosity determinations and formal titrations recorded.

Two susceptible strains and one resistant strain of butyl organisms were tested, A211, 101, and FEB. The effect of the sluggish principle was tested in the presence of a susceptible culture and in a control flask. Other control determinations included the plain control flasks testing the media for sterility and constancy during the experiment, a control flask containing a known liquifier Flavobacterium suaveolens, and another flask containing a known non-liquifier, Escherichia coli.

All cultures were grown at 37° C.; even though that is above the optimum for the Flavobacterium suaveolens. The butyl organisms were grown in anaerobic jars repeatedly evacuated until the medium began to boil and then was flooded with nitrogen. Traps were attached to liberate fermentation gases. The butyl cultures could be grown under these conditions in glucose gelatin even though the inoculation ratio was less than 0.05%, or two platinum loops of corn mash culture in 200 cc. The toxic filtrate was added in only small amounts, two drops for 200 cc.

The butyl organisms did not grow in the plain gelatin. The medium remained perfectly clear, there was no evidence of gasing and the acidity remained constant. After the experiment was completed a small portion of the medium was inoculated into corn mash and it was found that all flasks except one produced growth in corn mash showing that viable cells existed throughout the incubation period.

Sorenson's formal titration, chosen by Levine and Carpenter (29) as a convenient method of detecting the hydrolysis of the gelatin is really a measure of the carboxyl groups rather than the amino groups, because the increase in acidity is determined after the basic amino groups are "muzzled" with formaldehyde. The technic used was to titrate 5 cc. of medium to the neutral point of phenolph-

thalein with N/10 NaOH. Then 10 cc. of neutralized 35% - 40% formaldehyde solution were added and the mixture allowed to stand at room temperature for 20-30 minutes, followed by titration to the neutral point for phenolphthalein. The second titration value was a measure of the amino acids present. Even though the formaldehyde solution was nearly neutralized a blank was always determined and subtracted.

The butyl organisms did not produce any appreciable increase in the formol titration (See Fig. IX) either normally or in the presence of the toxic filtrate.

Escherichia coli flasks did not increase in formol titration, but the Flavobacterium suaveolens produced a titration value of 7.80 cc. of N/10 NaOH for 5 cc. of medium in both glucose and plain gelatin.

An Ostwald viscosimeter was used at 40.0° for the viscosity studies. The gelatin was rejuvenated as suggested by Levine and Shaw (30) before each measurement which kept the control flasks constant for more than 100 hrs. and which was helpful in removing the dissolved gases. The gelatin solutions gave very satisfactory viscosity readings and the only precautions observed were to rejuvenate a sample and then pipette off 5 cc. of the supernatant solution for the viscosity measurement. Several readings

were taken on the same solution and in nearly all cases duplicate readings checked within 0.1 second and the least precise readings varied only 0.3 second apart. The specific gravities of the liquids were constant between the limits  $1.003 \frac{40^{\circ}}{40^{\circ}}$  and  $1.008 \frac{40^{\circ}}{40^{\circ}}$  at all times. Therefore the time of flow in the viscosimeter was plotted in Fig.X instead of relative viscosity. The viscosity curves obtained on the three strains of butyl organisms and the strain in the presence of the sluggish principle were nearly identical and the general form of the curves was the same as those found by Stahly and Werkman (51) for the group of butyl organisms liquifying gelatin. The flask containing two drops of the toxic filtrate was identical with the control and the Escherichia coli flask was very nearly the same as the control flask. The Flavobacterium suaveolens was only slightly more effective in reducing the viscosity of the glucose gelatin solutions than the butyl organisms.

There was a noticeable decrease in the viscosity of the plain gelatin solutions even though the butyl organisms failed to grow. The results with the butyl strains were much more irregular in the plain gelatin than in the glucose gelatin. The growth in the glucose gelatin was nearly identical with all the strains tested. If the groups of Levine and Carpenter (29) are used, these butyl strains which are probably of the species Clostridium acetobutylicum

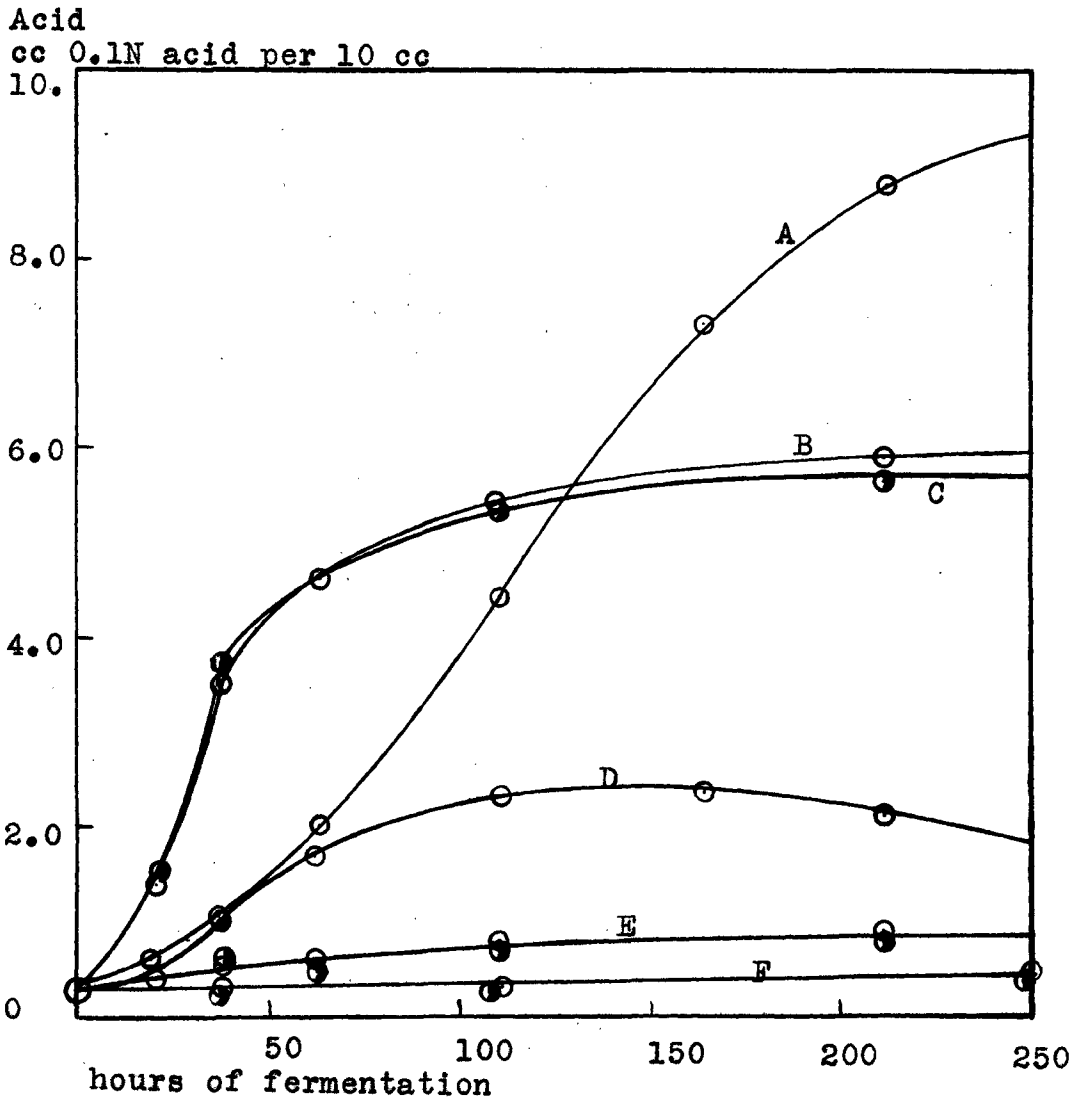


Fig. 9. Formol Titrations and Acidities in Glucose Gelatin.

A. and D. Formol titration and acidity of Flavobacterium suaveolens. 37°C.

E. and B. Formol titration and acidity of a normal fermentation. 37°C.

E. and C. Formol titration and acidity of a sluggish fermentation. 37°C.

F. Control, and a control plus 2 drops of toxic filtrate.

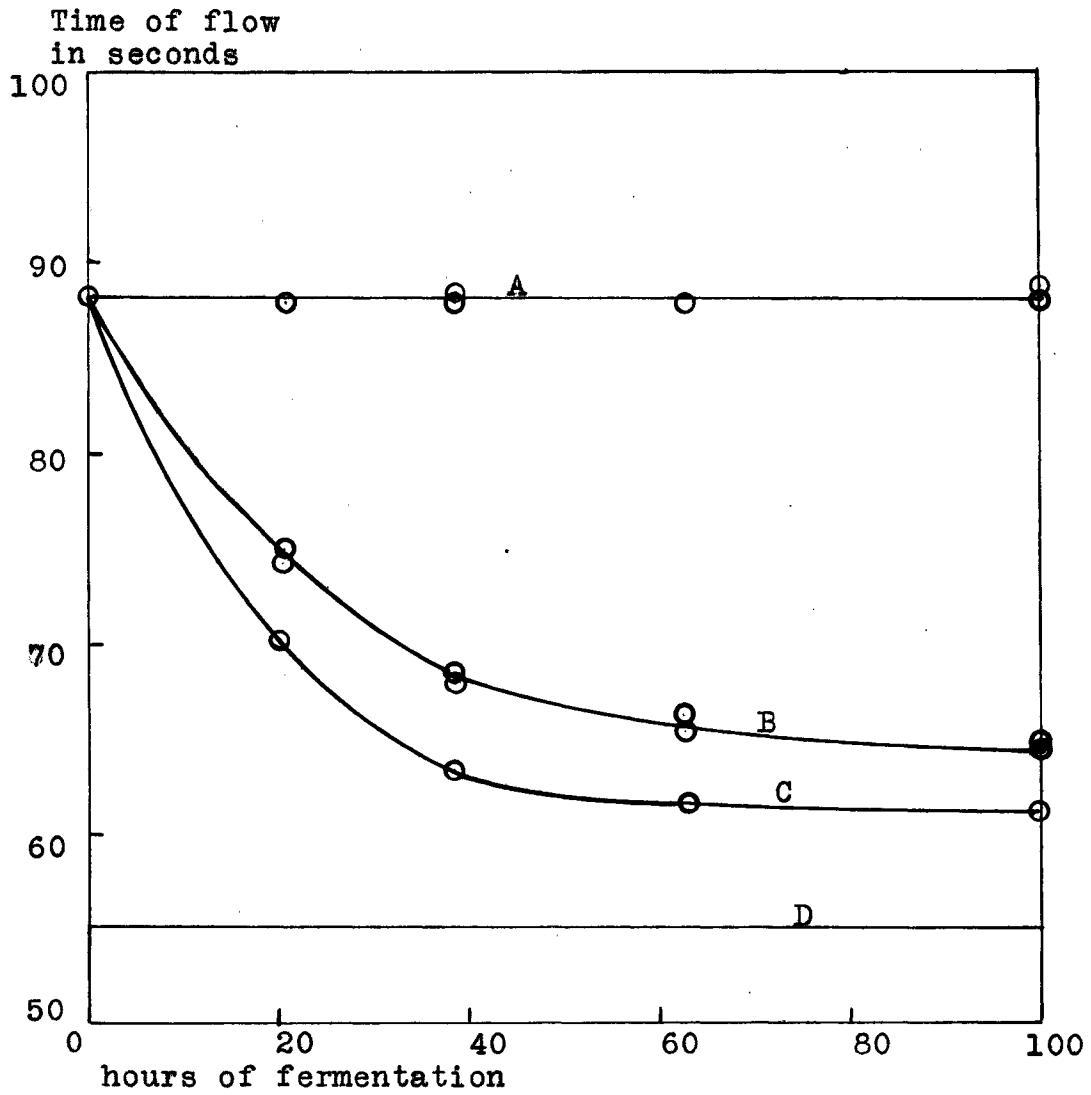


Fig. 10. Viscosities in Glucose Gelatin at 40°C.

- A. Control and control plus 2 drops of toxic filtrate.
- B. Normal fermentation and sluggish fermentation. 37°C.
- C. Flavobacterium suaveolens.
- D. Viscosity of water.

as characterized by McCoy and others (31), belong in the intermediate group including those species which decrease the viscosity of the gelatin medium but do not significantly change the formol titration. It is further evident that the decrease in the viscosity of the gelatin is probably not a step in the preparation of food material since the viscosity of the plain gelatin is decreased even though it is apparent that the bacteria were unable to grow and use it as food material. The addition of 2% glucose to the medium produced vigorous fermentation. It seems well that a group has been reserved for organisms such as these which certainly attack the gelatin, but still do not appear to use it as a source of food supply nor break it up into amino acids.

Legg (27) has suggested that the filterable sluggish principle might be a bacteriophage and it might therefore be expected to produce lysis of the bacterial cells. The tests were patterned after those described by d'Herelle (23). A clear broth was chosen for culturing the butyl bacteria similar to that reported by Weyer and Rettger (52).

It had the following composition :

15 g. malt extract (Bacto)  
30 g. sucrose  
1 g.  $K_2HPO_4$   
1 L. water



The butyl organisms grew vigorously in this malt extract broth and small colonies could be obtained by adding 1.5% agar to the above broth and incubating the plates in an anaerobic jar or using the inverted cover technique of Krumweide and Pratt (26). To test the lytic effect of the toxic filtrates, tubes were inoculated with 1 drop, 10 drops, and 20 drops of filtrate and then inoculated with a butyl culture, and observed closely. After twenty-four hours plates were made from each of the above tubes and from a control tube. Hanging drop mounts of the broth cultures were observed but there was no evidence of lysis either microscopically or macroscopically in the broth cultures. The tubes remained turbid until the organisms finally settled to the bottom. The bacterial colonies on agar were examined with the low power objective of the microscope but no lysis was observed. The normal colonies and the filtrate colonies appeared identical.

Heating the toxic filtrates either attenuated or destroyed the sluggish principle. The experimental evidence was obtained by inoculating several uniform tubes of 5% corn mash with a toxic filtrate. All of the tubes except two were plunged into a large boiling water bath and then two tubes were removed, cooled quickly at the following intervals, 1, 2, 4, 6, 8, 10, 13, 15, and 20 minutes.

All of the tubes were then inoculated with a vigorous 24 hour susceptible culture (Strain A211). Two normal tubes were prepared for comparison. The heads on the tubes were observed at 20 hrs., 24 hrs., and 40 hrs. and in some cases the final acidities were determined as a check. The results of the experiment described were fairly conclusive. The tubes heated 8 min. or longer in a boiling water bath produced excellent heads, while tubes heated 6 min., or less, developed only fair or poor heads. Apparently the sluggish principle in 5% corn mash tubes was destroyed or attenuated in 6 - 8 minutes time in a boiling water bath. The experiment has been repeated several times with different filtrates and it seems that 2 - 4 min. time in a boiling water bath is usually sufficient to eliminate the effect of a toxic filtrate in a tube of 5% corn mash. In one particular experiment a well defined difference was observed between a 2.0 min. and a 2.5 min. interval in the boiling water bath. The final acidities were also distinctly different and they confirmed the conclusions based on the observation of the heads.

The above experiments were repeated subjecting the toxic filtrate to heat shock at various pH values. A flask of about 500 cc. of 5% corn mash was sterilized in the autoclave at 15 pounds for two hours and then sterile,

dilute HCl was added to produce the desired pH value at 25° C. The corn mash was pipetted aseptically and uniformly into sterile tubes, two drops of filtrate added to each tube and then the tubes were heated in a boiling water bath for varying periods of time, 1, 2, 3, 4, 6, 8, 10, 12, 15, and 20 min. Two tubes were titrated and an equivalent of sterile standard NaOH added to each of the other tubes to bring the pH of the mash to 6.5 or 7.0 before inoculation with the butyl organism. The results are given in Table X.

TABLE X

EFFECT OF PH ON THERMAL DEATH TIME

pH of medium before heating	:	thermal death time in boiling water bath
4.0	:	4 - 6 min.
4.4	:	2 - 4 min.
5.1	:	2 - 4 min.
5.5	:	2 - 4 min.
6.8	:	2 - 4 min.

At 60° in an air bath the sluggish principle was destroyed with 40 - 60 min. exposure. The method used is not sufficiently precise to detect significant differences in the thermal death time at the pH values used, but the method did permit a qualitative or semi-quantitative study of the thermal resistance of the sluggish principle.

In order to determine more clearly the conditions which prevailed within the corn mash tube during the heat shock experiments, a thermometer was inserted in a tube of mash so that the bulb was in the center of the tube. The tube was heated in boiling water for varying periods of time and the temperature observed. To read the thermometer the tube was removed from the bath and the time and the highest point which the mercury reached was recorded. The tube was cooled to about 27 - 28° before heating again. Using the same tube duplicate determinations checked within 0.5° after 2 minutes heating and 1.0° after 0.5 minutes heating. A second series of values was obtained at another time on an entirely different batch of mash in another tube but the greatest difference is about 3°. The graphic representation of the results is shown in Fig. 11 while the experimental data is listed in Table XI.

The heating curves indicate that the general relationship  $\log \Delta T = -KM + \log (98 - 27)$  for a linear

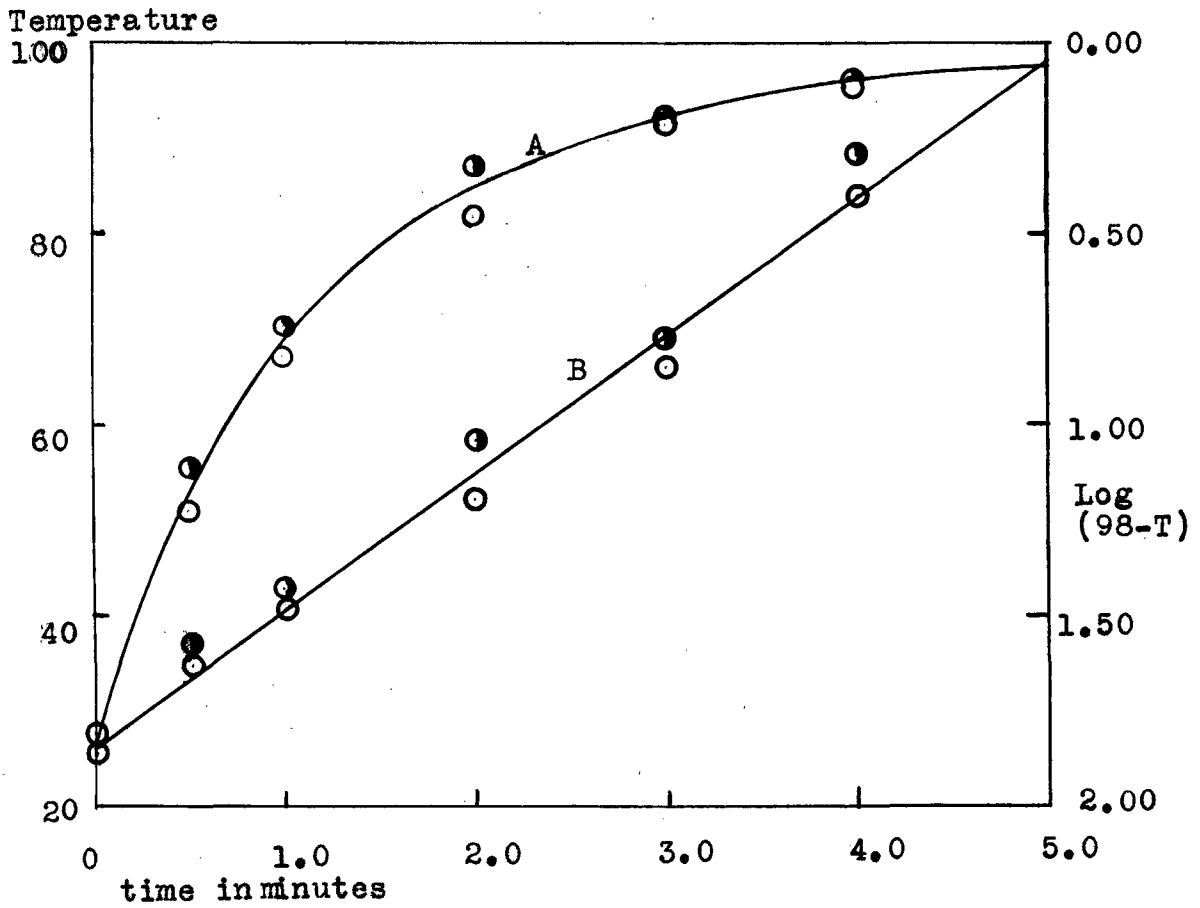


Fig. 11. Curves Representing the Heat Transfer from a Boiling Water Bath (98°C.) to the Interior of Corn Mash Tubes.

A.  $T = 98 - 10(1.85 - .4M)$

B.  $\text{Log}(98 - T) = -KM + \text{log}(98 - 27)$

TABLE XI

A STUDY OF HEAT TRANSFER FROM A BATH 98° C. TO THE  
INTERIOR OF CORN MASH TUBES

	M	T	T. calc.	98 - T	Log (98-T)	K
Tube 1						
2/24/33	0	27°	27	71	1.851	-
	0.5	55	53	43	1.643	.43
	1.0	67	70	31	1.492	.36
	2.0	82	87	16	1.204	.32
	3.0	91	93.5	7	.845	.34
	4.0	95.5	96.2	2.5	.398	.36
	6.0	97.5	97.7	0.5	.301	<u>.36</u>
					Average	<u>.36</u>
	0	27.	27	71	1.851	-
	0.5	51	53	37	1.568	.57
	1.0	70.5	70	27.5	1.440	.41
	2.0	87	87	11	1.042	.41
	3.0	92	93.5	6	.778	.36
	4.0	96	96.2	2	.301	<u>.39</u>
					Average	<u>.43</u>

$$\text{Log } \Delta T = \text{Log } (98 - T) = -K M + \text{log } (98 - 27)$$

$$K = 1/M [1.851 - \text{log } (98 - T)]$$

Using 0.4 as an average value for K and 27° C. for T° the experimental relationship,  $T = 98 - 10 (1.85 - .4M)$

may be derived. The equation was checked by comparing the observed T and the T calculated in Table XI.

equation is approximately correct for periods of time up to 5 or 6 minutes. The term ( $\Delta T$ ) is the difference between the temperature of the bath,  $98^{\circ}$ , and the temperature ( $T$ ) in the tube after ( $M$ ) minutes in the bath. The term  $K$  is a constant which is a function of the mash concentration, the thickness of the tube walls and any other factors which effect the rate of the heat transfer.

The results from the experiments regarding the thermal death point of the sluggish principle suggest that part of the advantage in heat shocking spores of a butyl culture when starting a fermentation may be due to the destruction of the sluggish principle which may have contaminated the culture or the medium. The time frequently used is two or three minutes in a boiling water bath or 10 minutes at  $80^{\circ}$  which would be very nearly the time required to destroy the sluggish principle.

The filtrates prepared at several different pH values during the study of the effect of pH on filtration were tested three months later in order to determine the effect of varying H - ion concentrations on the keeping of the sluggish principle. The tubes were stored in a cupboard at  $22^{\circ}$ -  $27^{\circ}$  C. As may be seen in Fig. 2 the various filtrations were nearly constant in virulence except for the ones filtered at pH 4.35 and 4.40. The pH values of the filtrates after filtration were the ones plotted

in Fig. 12 since it was at these H - ion concentrations that the sluggish principle was stored.

The filtrate tests were made in 300 cc. Erlenmeyer flasks containing 200 cc. of 5% sterile corn mash. The final acidities at 70 hrs. were taken as the measure of the virulence of the filtrate and were plotted against the storage pH in Fig. 12. That curve shows distinctly that the filtrate prepared and kept above pH 5.3 to pH 7.6 are more virulent than those prepared and kept at lower pH values. There are evidences of an optimum at pH 5.60.

Two different experiments were made to determine the effect of the radiation of a mercury vapor lamp on filtrate exposed in a vitrosil tube and in a Corex flask were rather indefinite. The heads observed indicated that the ultra-violet light from a mercury vapor lamp operated at 3.5 amperes and 18 inches from the flask of filtrate partially destroyed the sluggish principle after one hour's exposure, but the final acidities all were higher than might be expected normally.

Dilution experiments were performed with toxic filtrates in order to determine the number of units of the sluggish principle in a cubic centimeter of the filtrate. The dilution values were not always clear cut but 1000 units per cc. could be definitely demonstrated and over 100,000 units per cc. seemed to exist in some filtrates.



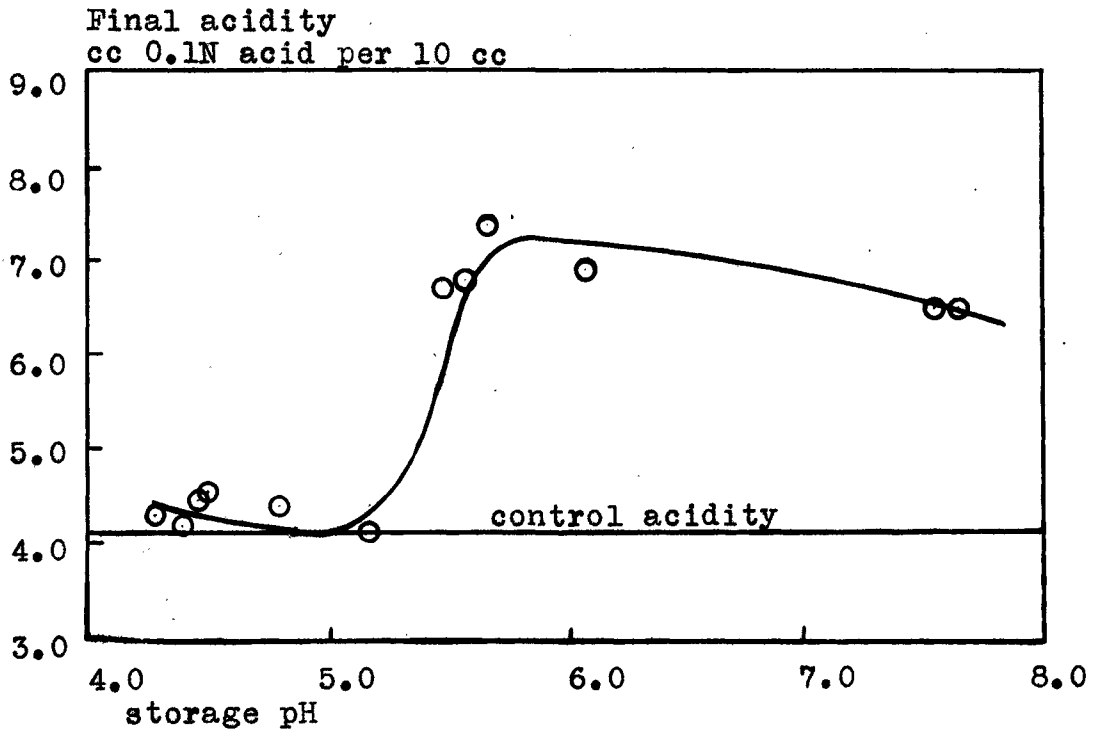


Fig. 12. Effect of pH on Storage at Room Temperature  
22-27°C.

Table XII lists a set of data obtained during one of the dilution experiments.

TABLE XII

Filtrate dilution	:	Head 28 hrs.	:	Final acidity 88hrs.
1 cc. - 1,000	:	NH	:	5.2      5.9
1 cc. - 100,000	:	EH	:	3.8      4.2
1 cc. - 10,000,000	:	EH	:	3.7      4.0
Controls	:	EH	:	3.8      4.0

The above test indicates definitely enough that there were between 1000 and 100,000 units of the sluggish principle per cc. in the above filtrate. The head formation and the final acidities were in agreement. Semi-quantitative determinations of the concentration of the sluggish principle in filtrates may be made in this manner.

#### SUMMARY AND CONCLUSIONS

In a sluggish fermentation there is occasionally a filterable substance which may be continually reproduced and collected in the presence of a susceptible butyl-acetonic fermentation. The suggestion has been made that this virulent material may be a bacteriophage.

The properties of the sluggish principle have not been determined completely enough to adequately define the material, but since it has not been demonstrated in normal cultures made sluggish with acid,  $\text{CaCO}_3$ , or continued transfer it is not to be derived from the bacteria themselves. The fact that the material can multiply and that small amounts to a certain limit are as effective as large amounts indicate that the material is autogenic. The material is apparently destroyed almost immediately by temperatures of  $80^\circ$  to  $90^\circ$  C. and in 40 to 60 minutes at  $60^\circ$ . Storage in solutions of pH less than 5.3 markedly deteriorates the substance. There is qualitative evidence leading to the belief that there is an optimum oxygen relationship. Therefore it seems helpful to think of the sluggish principle as a living parasitic material as Legg suggested.

Whether or not the material should be classed as a bacteriophage may be definitely determined in the future but at present the sluggish principle has not been observed to produce lysis of the bacterial cells and therefore does not definitely belong in the group termed bacteriophage.

The action of the sluggish/<sup>principle</sup>is to retard all of the normal functions of the bacteria especially the mechanism whereby the acids are converted to neutral products. The final result then depends upon whether the conversion of acids to neutral products occurs before the inhibiting acidity and H<sup>+</sup> ion activity is reached. Where the action of the sluggish principle is only mild the acidity peak is higher and broader than in a normal fermentation but the bacteria finally become active enough in converting the acid to neutral products so that the acid is utilized slightly faster than it is formed; the acidity finally drops and the fermentation gradually becomes more rapid and a nearly normal yield of solvents may be produced. If the sluggish principle is virulent enough, the conversion of acid to neutral products is delayed so long that the acid formed becomes inhibitory to all of the functions of the bacteria and the fermentation finally stops with only part of the carbohydrate used and without any decrease of the acid content.

Certain controlling factors in the development of sluggishness in the butyl-acetonic fermentation have been studied. Much needs to be done in further exploring this interesting phenomenon. If the data presented here should prove helpful in future studies, the purpose of this thesis will have been fulfilled.

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