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A study of some lipolytic microorganisms isolated from dairy products

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NOTE TO USERS

A STUDY OF SOME LIPOLYTIC MICROORGANISMS ISOLATED FROM DAIRY PRODUCTS

by

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TABLE OF CONTENTS

Page

INTRODUCTION

The study of fat hydrolysis by microorganisms has been greatly stimulated by the introduction of the nile blue sulfate technic. Although the technic, as ordinarily employed, possesses a certain disadvantage due to the toxicity of the dye, it is nevertheless very useful because of its simplicity and the clearness of the reaction obtained. Using this procedure investigators at the Iowa Agricultural Experiment Station have shown that fat is attacked by various groups of organisms found in milk. With certain of these organisms, for example Pseudomonas fragi, fat hydrolysis is an outstanding characteristic while with others, such as Alcaligenes viscosus, the hydrolysis of fat is a little known characteristic and the organism is best known for the production of ropiness in certain dairy products.

It appears that the ability of an organism to hydrolyze fat is an important character and, in the future, will be considered along with the other biochemical features in preparing descriptions of species. The need for simple and reliable methods of determining lipolysis is therefore apparent. It is also evident that the various methods of detecting lipolysis may not be equally useful for different purposes.

In the course of the present investigation many lipolytic cultures have been isolated. Certain of these have already been described more or less completely, while others apparently

have never been studied. It is evident that, to the dairy bacteriologist, adequate descriptions of the organisms bringing about changes in the fat of dairy products are essential if progress is to be made in understanding and eliminating these changes.

STATEMENT OF PROBLEM

The purposes of the present investigation were:

1. To consider a number of the methods used for the investigation of bacterial lipolysis.

2. To isolate and study a number of lipolytic microorganisms commonly found in dairy products.

3. To prepare adequate descriptions of certain of the lipolytic microorganisms found in order to aid in their identification.

4. To determine the effect of pure cultures of certain lipolytic organisms on various dairy products.

METHODS

Detection of Bacterial Lipolysis

The Nile Blue Sulfate Technic

The method used for the nile blue sulfate technic differed slightly from that proposed by Hammer and Collins (15). Beef infusion agar adjusted to a pH of 6.8 was used throughout. Before pouring the agar 0.5 ml. of a sterile emulsion of 2 per cent cottonseed oil in 0.5 per cent agar was added to each plate. A sterile aqueous solution of the dye was added to the melted agar in a sufficient quantity to give a final concentration of 1 part dye to 10,000 parts of agar. Generally this was accomplished by adding 5 ml. of a 1 to 500 solution of the dye to 95 ml. of the melted agar. The medium used will be referred to as the nile blue sulfate medium and the technic as the nile blue sulfate technic.

The Modified Nile Blue Sulfate Technic

The modified nile blue sulfate technic was carried out according to the procedure outlined by Long and Hammer (19). Before pouring with beef infusion agar, 0.5 ml. of a sterile emulsion of 2 per cent cottonseed oil or other fat in 0.5 per cent agar was added per plate. After incubation the plates were flooded for 30 minutes with a 1 to 1,500 aqueous solution of nile blue

sulfate and then rinsed with distilled water. Lipolysis was indicated by a change in appearance of the fat globules; those in the vicinity of lipolytic colonies were stained blue and those at a distance were stained pink.

The Simple Triglyceride Technic

The general procedure outlined by Anderson (1) was used for the simple triglyceride technic. However, in order to obtain proper dispersion of the triglycerides in the medium, emulsions of them were prepared. A satisfactory method was to add 0.5 ml. of an emulsion of 3 to 4 per cent of tripropionin or tributyrin in 0.5 per cent agar to each plate before pouring with beef infusion agar. A disappearance of the globules in the vicinity of a colony was considered evidence of hydrolysis.

The Natural Fat Technic

The natural fat technic consisted of the addition of 0.5 ml. of a sterile emulsion of 2 per cent cottonseed oil or butter fat in 0.5 per cent agar to each plate before pouring with beef infusion agar. Hydrolysis of the fat was indicated by the globules becoming whiter and more opaque in the vicinity of lipolytic microorganisms.

Numbers of Organisms in the Samples Examined

The total and lipolytic counts were recorded on the basis of the number of microorganisms per milliliter. In many cases it was necessary to refer to the number of lipolytic organisms in a sample as less than a certain number per milliliter. This may indicate either that there were none present or that the proportion of lipolytic organisms to the total number of organisms present in the sample was small and the lipolytic bacteria were consequently diluted out in the plates suitable for examination. Frequently, however, a lower dilution than that used for the total count can be used for the lipolytic count and quite satisfactory results can be obtained. Occasionally in a comparatively large area where the fat globules are all hydrolyzed it may be difficult to detect whether the lipolysis was caused by one or several colonies.

Type of Lipolysis

The type of lipolysis was studied using the procedure suggested by Collins and Hammer (8). Uninoculated plates were prepared with the nile blue sulfate technic. After the agar had solidified and the surface had become relatively dry, spot inoculations were made with the pure cultures to be investigated. The plates were held at 21° C. and examined after various periods for "complete" or "incomplete" lipolysis, the term complete

indicating that all of the fat globules below the bacterial growth had been hydrolyzed and the term incomplete indicating that only a part of the globules beneath the growth had been attacked. Observations on the presence of a readily diffusible lipolytic enzyme were also made; these were based on whether or not the zone of lipolysis extended for a considerable distance from the colony. Cottonseed oil was used throughout the study for the final determination of the lipolytic abilities of the various cultures isolated.

Hydrolysis of the Simple Triglycerides Other than Tripropionin and Tributyrin

The ability of the cultures to hydrolyze the simple triglycerides, other than tripropionin and tributyrin, was determined as follows: a small quantity of the triglyceride to be tested was added to a tube of agar containing nile blue sulfate in the proportion of 1 to 10,000 and the tube was agitated vigorously to properly disperse the compound. The mixture was then poured into a petri dish and allowed to solidify. After the agar was dry the cultures were spotted on it; following incubation the plates were examined. Hydrolysis was indicated by a change in the color of the triglyceride.

Action of the Cultures on Butter

In studying the action of an organism on butter sweet cream

was pasteurized at 82° C. for 15 minutes, cooled, and placed in sterile one quart glass jars in approximately one pint amounts; the cream was then inoculated with a young litmus milk culture of the organism to be tested and churned. The butter was worked, using sterile equipment, and stored in sterile containers at 21° C. without the addition of salt. In some instances portions of the butter were also stored at approximately 5° C.

Production of Acetylmethylcarbinol Plus Diacetyl

The acetylmethylcarbinol plus diacetyl was determined in skim milk cultures according to the procedure followed by Michaelian and Hammer (20)

Volatile Acidity

The volatile acidity was studied using the procedure outlined by Michaelian and Hammer (20).

Tests Used in Preparing Descriptions of the Organisms

The production of indol, reduction of nitrates, fermentation of carbohydrates and hydrolysis of starch were determined with the methods outlined by the Committee on Bacteriological Technic of the Society of American Bacteriologists (9). The ability of the cultures to liquefy gelatin was studied with a 10 per cent nutrient gelatin. The production of hydrogen sulphide was

determined with the technic proposed by Levine et al (18).

The Voges Proskauer and methyl red tests were carried out on a medium of the following composition: glucose 0.5 per cent, proteose peptone (Difco) 0.5 per cent and dibasic potassium phosphate 0.5 per cent. The creatine modification was used in testing for acetylmethylcarbinol. A small amount of creatine and 5 ml. of a 40 per cent solution of sodium hydroxide was added to approximately 5 ml. of the medium. A red coloration indicated a positive reaction. The methyl red solution was prepared by adding 0.1 gm. of methyl red to 300 ml. of 95 per cent alcohol and diluting with water to 500 ml. Five drops of this solution were added to 5 ml. of medium. A red coloration indicated a positive test and a yellow one a negative test.

Hemolysis was studied by streaking or spotting cultures on plates prepared by adding 0.5 ml. of defibrinated sheep blood per plate before pouring with beef infusion agar.

EXPERIMENTAL

SECTION I. OBSERVATIONS ON THE METHODS USED FOR THE DETECTION OF BACTERIAL LIPOLYSIS

Various methods have been employed for the study of bacterial lipolysis. The general technics followed were reviewed by Collins and Hammer (7) in 1934. Section I deals with the procedures that have been most widely employed in recent investigations.

The Nile Blue Sulfate Technic

The nile blue sulfate technic consists essentially of adding nile blue sulfate and some natural fat, such as cottonseed oil or butter fat, to agar that is used for pouring plates or that is inoculated after solidification. Fat globules at a distance from lipolytic colonies are stained pink while those in the vicinity of lipolytic colonies are stained blue. The procedure employed in this study is essentially that of Hammer and Collins (15) with a few modifications.* The nile blue sulfate technic is simple and the results are easily read. There is no difficulty in detecting the difference in color when a fat globule is hydrolyzed, since the globules change from a distinct pink to a distinct blue. The medium is well adapted to the isolation of lipolytic colonies as the dye is added to the

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*The details of the procedure used are given in the "Methods".

agar at the time of plating and the picking of desired organisms is not complicated by flooding the plates. The primary disadvantage of the technic is that certain organisms, especially cocci, are inhibited by the dye. However, it appears that cocci are not strongly lipolytic and do not often cause rancidity in dairy products. If this is true the inhibitory action of the dye may be an advantage, due to the fact that the medium has a selective action. Thus, in certain instances, the isolation of fat hydrolyzing cultures is made possible when the lipolytic organisms are present in small numbers as compared to the total organisms.

Effect of the Temperature of Incubation on the Total and Lipolytic Counts Obtained on the Nile Blue Sulfate Medium

The effect of the temperature of incubation on the total and lipolytic counts obtained on the nile blue sulfate medium was investigated by plating, in duplicate, samples of the milk supplied to Iowa State College by various producers. One set of plates was incubated at 21° C. for 4 days and the other at 37° C. for 2 days. The results obtained are given in table I.

In general there was a tendency to obtain somewhat higher total counts after 2 days at 37° C. than after 4 days at 21° C. In the 48 trials, the higher total count was obtained in 28 at 37° C. and in 19 at 21° C. while in 1 trial the counts

TABLE I

EFFECT OF TEMPERATURE OF INCUBATION ON THE TOTAL AND LIPOLYTIC COUNTS OBTAINED ON NILE BLUE SULFATE MEDIUM

Sample No.	Conditions of incubation			
	4 days at 21° C.		2 days at 37° C.	
	Bacteria per ml.		Bacteria per ml.	
	Total	Lipolytic	Total	Lipolytic
1	7,000	100	11,100	100
2	4,600	200	12,500	< 100
3	14,000	< 1,000*	18,000	< 1,000*
4	2,100	< 100	3,400	< 100
5	12,700	< 1,000*	23,000	< 1,000*
6	7,200	< 100	8,900	< 100
7	17,000	< 1,000	37,000	< 1,000
8	21,000	1,000	24,000	< 1,000
9	26,000	200	23,000	< 1,000
10	32,000	200	10,000	< 1,000*
11	72,000	< 1,000	76,000	< 1,000
12	600	100	500	< 100
13	5,800	< 100	6,500	< 100
14	400	< 100	1,500	< 100
15	9,000	< 100	1,000	< 100
16	100	< 100	400	< 100
17	500	< 100	3,800	200
18	1,100	< 100	1,900	< 100
19	1,100	< 100	24,000	< 1,000
20	2,400	< 100	5,200	< 100
21	600	< 100	< 100	< 100
22	360,000	<10,000	311,000	< 1,000
23	35,000	100	58,000	100
24	309,000	< 1,000	340,000	<10,000
25	49,000	200	34,000	< 1,000
26	109,000	300	110,000	< 1,000
27	16,000	< 1,000*	34,600	< 100
28	784,000	300	411,000	< 1,000
29	51,000	1,000	45,000	< 1,000
30	27,000	100	16,000	100
31	8,600	8,000	300	< 100
32	32,000	23,000	4,400	300
33	5,200	200	4,900	< 100
34	3,700	< 100	600	< 100
35	2,600	< 100	3,100	< 100
36	800	< 100	500	< 100
37	1,000	< 100	5,800	< 100

TABLE I continued

Sample No.	Conditions of incubation			
	4 days at 21° C.		2 days at 37° C.	
	Bacteria per ml.		Bacteria per ml.	
	Total	Lipolytic	Total	Lipolytic
38	100	< 100	400	< 100
39	230,000	60,000	320,000	10,000
40	114,000	4,000	205,000	14,000
41	376,000	5,000	560,000	2,000
42	197,000	< 1,000	189,000	< 1,000
43	5,000	< 100	3,100	100
44	2,100	< 100	3,600	600
45	5,200	< 100	10,000	< 1,000*
46	39,000	500	28,000	100
47	70,000	7,000	29,000	100
48	1,100	< 100	1,100	< 100

*In a few instances, due to the presence of spreaders or to the fact that plates were unsuitable for lipolytic counts, higher dilutions were used in determining the lipolytic count than were used in determining the total count.

obtained at the two temperatures were equal. However, definite differences in the total counts obtained on the nile blue sulfate medium at the two temperatures were infrequent. In general, lipolytic counts were low at either temperature. There was a tendency, however, to obtain somewhat higher counts when the plates were incubated at 21° C. than when they were incubated at 37° C. As the nile blue sulfate technic is intended primarily for the study of lipolysis the data indicate that the lower incubation temperature should be used.

The Modified Nile Blue Sulfate Technic

As stated previously the nile blue sulfate technic is very satisfactory for certain organisms but for others (notably cocci) it is definitely inhibitory. For the latter types some other method or some modification of the nile blue sulfate technic is desirable if total counts are to be made. Long and Hammer (19) proposed a modification of the nile blue sulfate technic which avoided inhibition of growth due to action of the dye. The organisms are grown on plates containing dispersed cottonseed oil or butter fat and, after incubating 5 days at 21° C., the plates are flooded for 30 minutes with an aqueous solution of nile blue sulfate of an approximate strength of 1 to 1,500. They are then rinsed with distilled water and observed for lipolysis. The procedure is essentially the same as that used

by Berry (3) and first proposed by Carnot and Mauban (5), except that nile blue sulfate solution is used as an indicator instead of a saturated aqueous solution of copper sulfate.

A study was made of the comparative counts obtained with the nile blue sulfate technic and the modified technic as proposed by Long and Hammer (19). The milk supplied to Iowa State College by various producers was plated at intervals extending over a period of approximately 2 months. In all instances the plates were incubated at 21° C. for 5 days. The data are given in table II.

The results indicate that nile blue sulfate had a decided effect on the total counts. In all of the 58 trials the total counts obtained with the modified technic were considerably higher than those obtained with the technic as ordinarily employed. As was the case with the total counts, the lipolytic counts obtained were also much higher with the modified nile blue sulfate technic. In only one instance out of 55 trials with which comparisons were possible was a higher lipolytic count obtained with the nile blue sulfate medium.

The results obtained indicate that the modified nile blue sulfate technic is relatively satisfactory and that its use will result in a more accurate estimate of the number of lipolytic organisms present in a sample. The modified technic gives a much higher total count and lipolytic count than the technic involving the addition of nile blue sulfate to the agar

TABLE II

A COMPARISON OF THE TOTAL AND LIPOLYTIC COUNTS OBTAINED WITH THE NILE BLUE SULFATE TECHNIC AND THE MODIFIED NILE BLUE SULFATE TECHNIC

Plates incubated 5 days at 21° C.

Sample No.	Nile blue sulfate technic		Modified nile blue sulfate technic	
	Bacteria per ml.		Bacteria per ml.	
	Total	Lipolytic	Total	Lipolytic
1	100	< 100	1,400	300
2	100	< 100	10,500	500
3	900	300	45,000	2,000
4	100	< 100	11,000	< 1,000*
5	700	< 100	24,000	< 1,000
6	1,500	200	38,000	1,000
7	1,100	< 100	18,000	3,000
8	1,400	200	220,000	3,000
9	2,200	200	25,000	< 1,000
10	< 100	< 100	250,000	<10,000
11	100	< 100	12,000	< 1,000*
12	200	< 100	75,000	1,000
13	100	< 100	149,000	< 1,000
14	200	< 100	140,000	2,000
15	< 100	< 100	8,600	200
16	100	< 100	1,000	1,000*
17	6,800	200	60,000	< 1,000
18	< 100	< 100	16,000	2,000
19	< 100	100	13,400	6,000
20	400	100	50,000	6,000
21	200	< 100	7,900	100
22	1,600	< 100	34,000	20,000
23	700	< 100	43,000	1,000
24	700	< 100	82,000	1,000
25	< 100	< 100	18,000	5,000
26	500	< 100	46,000	2,000
27	336,000	24,000	890,000	20,000
28	600	< 100	65,000	<10,000*
29	800	< 100	92,000	
30	300	< 100	1,420,000	<10,000
31	500	500	14,200,000	300,000
32	400	< 100	8,000,000	320,000
33	< 100	< 100	142,000	
34	4,100	< 100	660,000	70,000
35	18,500	< 100	3,150,000	30,000

TABLE II continued

Sample No.	Nile blue sulfate technic		Modified nile blue sulfate technic	
	Bacteria per ml.		Bacteria per ml.	
	Total	Lipolytic	Total	Lipolytic
36	200	< 100	132,000	2,000
37	< 100	< 100	91,000	2,000
38	2,400	< 100	146,000	2,000
39	71,000	15,000	810,000	40,000
40	15,700	200	690,000	10,000
41	32,000	11,000	9,200,000	400,000
42	1,200	< 100	1,790,000	50,000
43	500	400	340,000	20,000
44	< 100	< 100	180,000	< 1,000
45	< 100	< 100	290,000	<10,000
46	4,000	100	960,000	30,000
47	1,728,000		2,980,000	
48	300	100	84,000	5,000
49	4,600	< 100	67,000	< 1,000
50	500	100	29,000	1,000
51	500	< 100	149,000	6,000
52	1,000	500	66,000	40,000
53	1,400	300	2,400,000	400,000
54	23,000	4,000	1,410,000	60,000
55	300	100	130,000	1,000
56	1,200	500	240,000	4,000
57	300	< 100	109,000	1,000
58	600	< 100	111,000	< 1,000

*See note table I.

at the time of plating. However, the flooding of plates after incubation is a disadvantage in that it complicates the picking of lipolytic colonies. This disadvantage may be overcome by several replatings of the colonies picked from plates flooded with the dye or by pouring a second set of plates to be used for purposes of isolation.

The Simple Triglyceride Technic

The simple triglyceride technic consists of the addition of tripropionin or tributyrin to agar used for growing organisms and the detection of lipolysis by a disappearance of the compound. The method depends on the fact that the products of hydrolysis of these triglycerides are soluble in the agar and thus a clear zone is produced in the vicinity of lipolytic colonies. This procedure, using tributyrin, was proposed by Anderson (1) as a means of detecting lipolytic bacteria. It should be noted that, due to the solubility of the compound, triacetin cannot be used in the technic and also that simple triglycerides higher in the series than tributyrin cannot be employed due to the fact that, in the event of hydrolysis, the fatty acids freed are only slightly soluble in the agar. Essentially the same procedure was used by Collins and Hammer (7) in a study of the action of certain bacteria, most of which were lipolytic, on some simple triglycerides but the method was

not advocated as a suitable one for the detection of lipolysis. These investigators found that tripropionin was the most easily hydrolyzed of all the triglycerides studied and that hydrolysis of the simple triglycerides of the saturated fatty acids was more difficult as the molecular weight of the acids increased. Triolein was hydrolyzed fairly easily.

While there is no doubt that bacteria capable of hydrolyzing natural fats will produce a clear zone around the colonies when inoculated on a plate containing dispersed globules of either tripropionin or tributyrin, there is strong evidence that certain organisms commonly regarded as non-lipolytic will also bring about the same reaction. Long and Hammer (19), in a study of Streptococcus liquefaciens, noted that certain of their cultures hydrolyzed tripropionin, tributyrin or both but gave no evidence of attacking either cottonseed oil or butter fat when tested by the modified nile blue sulfate technic. They accordingly regard S. liquefaciens as non-lipolytic.

Factors Affecting the Results Obtained in Using the Simple Triglyceride Technic

In studying the ability of certain organisms to hydrolyze tripropionin and tributyrin, a lack of uniformity in the results was experienced. Occasionally a culture would not give identical results on a triglyceride in different trials and the extent of hydrolysis often varied. Observations were made

on the causes of these irregularities and the following factors were considered to be of importance:

THE TYPE OF MEDIUM USED.

Unless the medium employed was one supporting maximum growth of the organism and allowing an ample opportunity to act on the triglyceride tested, the results obtained were sometimes indefinite.

THE SIZE AND DISTRIBUTION OF THE TRIGLYCERIDE GLOBULES IN THE MEDIUM.

It was noted that when the dispersed globules were too large and were unevenly distributed hydrolysis by certain cultures could not be detected. With a fine and even dispersion of the triglycerides in agar, the same cultures would readily cause the disappearance of the globules. This was evidently due to the fact that with the large globules the hydrolysis was insufficient to cause them to disappear.

THE DEPTH OF THE AGAR IN THE PLATE.

It was found essential to limit the thickness of the agar in the plates in order to facilitate detection of lipolysis. Even though an organism did hydrolyze a portion of the globules surrounding the colony, it was often impossible to determine this

because hydrolysis of the triglyceride did not extend through the thickness of the agar to the bottom of the layer.

Accordingly the thinest possible layer that would not dry out during the period of incubation was employed.

THE SUSCEPTIBILITY OF THE INDIVIDUAL CULTURES TO THE PRODUCTS OF HYDROLYSIS.

Certain cultures of a given species appeared to show a relatively slight growth on the plates containing a triglyceride. It is conceivable that these cultures hydrolyzed the triglyceride as they began to develop and being less resistant to the products of hydrolysis, their growth was stopped before that of other cultures that were not so susceptible.

THE TEMPERATURE OF INCUBATION.

The temperature of incubation was considered to be of importance in that any acid freed would likely be more toxic at a higher temperature that at a low one. Therefore a low temperature and long period of incubation were used in order to give the cultures maximum opportunity to act on the triglycerides.

Action of *Streptococcus lactis* on Tripropionin and Tributyrin

In order to obtain more information on the simple triglyceride technic as a method for studying bacterial lipolysis

the action of 32 S. lactis cultures of various origins on cottonseed oil, tripropionin and tributyrin was investigated. The cultures were first tested by the modified nile blue sulfate technic for their ability to hydrolyze cottonseed oil. As was expected none of them gave any evidence of attacking the fat. Each of the 32 cultures was then examined for its ability to hydrolyze tripropinonin and tributyrin by spotting on plates prepared according to the procedure described under "Methods". The plates were incubated 6 days at 21° C. and examined for evidence of hydrolysis. The data are presented in table III.

The results show that tripropionin was easily hydrolyzed by S. lactis, only two of the 32 cultures employed failing to attack the compound. Fourteen of the cultures brought about hydrolysis of all of the globules below the colonies and 15 hydrolyzed a part of them, one culture produced questionable hydrolysis below the colony while two did not show any indication of attacking tripropionin. Twenty-nine of the cultures showed hydrolysis around the colonies, one was questionable in this respect and two gave negative results.

Tributyrin appeared to be less easily hydrolyzed by S. lactis than tripropionin. Of the 32 cultures, six hydrolyzed all of the globules below the colonies, 12 hydrolyzed a part of them, five brought about questionable hydrolysis and nine gave no evidence of attacking tributyrin. Fifteen cultures formed a small zone of hydrolysis around the colonies, two were question-

TABLE III

ACTION OF STREPTOCOCCUS LACTIS ON TRIPROPIONIN AND TRIBUTYRIN

Culture No.	Hydrolysis of tripropionin		Hydrolysis of tributyrin	
	Below colony	Around colony	Below colony	Around colony
1	Questionable	Questionable	Negative	Negative
2	Incomplete	Positive	Questionable	Negative
3	Incomplete	Positive	Negative	Negative
4	Complete	Positive	Complete	Positive
5	Complete	Positive	Complete	Positive
6	Complete	Positive	Complete	Positive
7	Negative	Negative	Incomplete	Positive
8	Complete	Positive	Complete	Positive
9	Negative	Negative	Incomplete	Questionable
10	Complete	Positive	Complete	Positive
11	Incomplete	Positive	Questionable	Questionable
12	Complete	Positive	Incomplete	Positive
13	Incomplete	Positive	Incomplete	Positive
14	Complete	Positive	Incomplete	Positive
15	Complete	Positive	Incomplete	Positive
16	Incomplete	Positive	Questionable	Negative
17	Complete	Positive	Questionable	Negative
18	Incomplete	Positive	Negative	Negative
19	Incomplete	Positive	Incomplete	Positive
20	Incomplete	Positive	Incomplete	Positive
21	Complete	Positive	Incomplete	Negative
22	Incomplete	Positive	Incomplete	Negative
23	Complete	Positive	Negative	Negative
24	Incomplete	Positive	Incomplete	Positive
25	Complete	Positive	Negative	Negative
26	Incomplete	Positive	Incomplete	Positive
27	Complete	Positive	Complete	Positive
28	Incomplete	Positive	Negative	Negative
29	Incomplete	Positive	Negative	Negative
30	Incomplete	Positive	Negative	Negative
31	Incomplete	Positive	Negative	Negative
32	Complete	Positive	Questionable	Negative

able in this respect and 15 were negative.

The data reported indicates (a) the technic employing tributyrin as a test for lipolysis is not a reliable one and (b) the assumption an organism hydrolyzing tributyrin will also hydrolyze butter fat or cottonseed oil is erroneous. The results are in agreement with the work of Long and Hammer (19) who found that the ability of S. liquefaciens to hydrolyze tripropionin and tributyrin was not accompanied by an ability to hydrolyze the natural fats. It is also in agreement with the observation of Collins (6) who found that certain bacterial cultures could hydrolyze tripropionin and tributyrin but not butter fat. It agrees with his conclusion that tripropionin cannot be used for the detection of definitely lipolytic bacteria but does not support his conclusion that tributyrin can be used for the separation of lipolytic and non-lipolytic cultures.

The Natural Fat Technic

The natural fat technic consists of the addition of emulsified cottonseed oil to agar and the detection of lipolysis by a change in the appearance of the fat globules. During a comparison of the nile blue sulfate method and the modified method it was noted that around certain colonies on the plates containing fat, but no dye, the globules of fat were distinctly different in appearance than those at a distance from the colonies. The globules that differed were whiter, more opaque and appeared to

have been changed in some manner by the action of the bacteria. It was thought that in the process of hydrolysis fatty acids of a relatively high molecular weight were freed and, being solid at room temperature and insoluble in the agar, remained in the fat globule and changed its appearance. Accordingly, after flooding with nile blue sulfate, these areas were noted especially for lipolysis. It was found that when the globules having an opaque appearance were stained they were distinctly blue, indicating that the fat had been hydrolyzed. This suggested that the appearance of the hydrolyzed fat globules might be used as a test for lipolysis. Technics involving the addition of natural fat to agar have been proposed by earlier investigators (4, 11, 22) but in all cases a clearing of a zone around lipolytic colonies was relied upon as a test for hydrolysis.

In order to test the method of using a dispersed fat but no indicator in the agar, 54 pure cultures that were known to be lipolytic were spotted on plates containing dispersed cottonseed oil and also on plates containing dispersed butter fat. The plates were incubated for 5 days at 21° C. and examined with a 6 X binocular for lipolysis as evidenced by a change in appearance of the fat globules. Examination of the plates containing dispersed butter fat was found to be greatly facilitated by warming them in a 45° C. incubator just before the observations were made. This is due to the fact that at room temperature butter fat is solid and rather opaque in appearance.

The 54 lipolytic cultures employed hydrolyzed cottonseed oil when tested by the natural fat method. The results obtained by use of this method were in close agreement with those obtained when the nile blue sulfate technic was employed. When differences were observed they were only in the extent of lipolysis and it is entirely possible that such differences would be found if the same method was employed in several trials. With butter fat dispersed in the agar in place of cottonseed oil the results obtained agreed closely with those obtained on cottonseed oil; in no instance did a culture fail to show lipolysis.

During the routine plating of 48 samples of butter, a comparison was made between the lipolytic counts obtained by the natural fat technic and by the modified nile blue sulfate technic. At the time of plating 0.5 ml. of a 2 per cent cottonseed oil emulsion in 0.5 per cent agar was added per plate. At the end of the incubation period the unstained plates were examined for lipolytic bacteria. The plates were then flooded with a 1 to 1,500 aqueous solution of nile blue sulfate for 30 minutes, rinsed with distilled water and again examined for lipolytic colonies. With 42 of the samples no lipolytic colonies were observed. With 5 of the samples the results obtained by the two methods were in exact agreement; these samples included one that contained lipolytic molds but no lipolytic bacteria. The lowest dilution poured on the remaining sample contained a weakly lipolytic colony that was missed when the plate was examined

before flooding with nile blue sulfate. The natural fat method worked very well with strongly lipolytic bacterial cultures and particularly well with actively lipolytic molds. As the lipolytic ability of a culture decreased the detection of fat hydrolysis by this technic became more difficult.

The data obtained indicate that for the isolation of organisms inhibited by nile blue sulfate the natural fat technic may be useful. The method does not involve flooding the plates with a dye and the picking of lipolytic colonies is thus simplified. Since there is no material present to inhibit growth of the microorganisms it is possible to obtain a total count as well as a lipolytic count on one set of plates. Observations have shown, however, that by use of the natural fat technic it may be difficult or even impossible to detect weakly lipolytic colonies. In addition, if lipolytic microorganisms are present in small numbers, as compared to the total numbers, it is possible they may be diluted out in the plates used for total counts. In this case it is often practical to use a lower dilution for the lipolytic count than is used for the total count.

SECTION II. DISTRIBUTION OF LIPOLYTIC MICROORGANISMS IN DAIRY PRODUCTS

The work of various investigators has indicated that many species of bacteria possess the power of hydrolyzing fat. Certain of these, such as Serratia marcescens, have long been recognized as fat hydrolyzers but are not important in dairy

products, due to their infrequent occurrence. Other lipolytic organisms not as well known are encountered more or less regularly. An example of the latter group is Pseudomonas fragi which appears to be widely distributed and which is often the cause of defects in various dairy products. Since many species of microorganisms are known to be lipolytic, cultures capable of hydrolyzing fat would be expected to be present in various materials. Studies on the isolation of these types indicate that this is true. Lipolytic species have been isolated from a variety of dairy products, both normal and abnormal, and have also been isolated from other sources. Although lipolytic organisms are distributed widely in dairy products, in many instances they are present in relatively small numbers. As noted in the previous section the lipolytic count on a particular sample is often rather low in comparison to the total count and, as a consequence, the lipolytic organisms are diluted out in the plates suitable for counting. In order to detect these types various means may be employed, such as enrichment through the incubation of the sample at a low temperature or the use of a selective medium such as nile blue sulfate.

During the plating of numerous samples of dairy products and a considerable number of specimens of various other materials many cultures of lipolytic organisms have been obtained. Most of these were picked from plates prepared with the nile blue sulfate technic while a portion of them were picked from plates

prepared with the natural fat technic. In addition a number of the lipolytic cultures isolated were obtained from plates prepared for some other purpose than studying lipolytic bacteria; the ability of these cultures to hydrolyze fat was detected either by spotting them on nile blue sulfate medium or by studying their action in butter.

Milk

In the plating of many normal and abnormal samples of raw milk a large number of lipolytic organisms have been isolated. The normal milk examined was largely obtained from the producers supplying Iowa State College but a number of samples obtained from various other points in Iowa were also included. Lipolytic cultures were readily isolated from normal, low count milk and appeared to be distributed rather widely in the samples plated. The abnormal milk studied consisted mostly of samples obtained from Iowa State College and held at 5° to 10° C. for extended periods. These samples generally developed off flavors and odors suggesting those brought about by Ps. fragi. When such material was examined it was found that, in the majority of cases, the flora was largely made up of bacteria of this type.

The plating of bottled pasteurized milk, using the nile blue sulfate technic, frequently yielded cultures of lipolytic

bacteria. Although in a few instances lipolytic cultures were isolated from apparently normal pasteurized milk, the majority of the cultures were obtained from milk that had been held for some time at relatively low temperatures and was abnormal in some respect. These samples generally contained Ps. fragi in relatively high numbers.

In order to investigate the possibility of lipolytic micro-organisms surviving pasteurization exposures, a number of trials were carried out in which lipolytic counts were made on milk taken from the pasteurizing vat immediately before and immediately after pasteurization. It was found that lipolytic organisms were frequently present in the milk before heating but in no instance were they isolated from milk obtained from the vat just after pasteurization. A number of the samples of milk taken from the vat immediately after pasteurization were also held at approximately 5° C. in order to obtain a further check on the presence of lipolytic organisms. The samples commonly showed no pronounced change after an extended period and lipolytic organisms were not present. These results suggest that the lipolytic organisms so frequently found in bottled pasteurized milk are due to contamination subsequent to processing, rather than to the organism surviving the pasteurization.

Cream

A considerable number of samples of cream obtained from

Iowa State College and from various plants in Iowa were plated on nile blue sulfate medium. As would be expected many lipolytic organisms were isolated and, due to the fact the different lots of cream were held under different conditions before being received, many types of organisms were secured. Species almost never encountered in the routine examination of milk may be obtained occasionally from cream. In several instances cultures of S. marcescens have been obtained and in at least one instance Pseudomonas mucidolens has been isolated from raw cream.

Frequently lipolytic cultures were obtained from samples of pasteurized cream that were defective. Many of them were definitely rancid while others possessed an odor and flavor suggesting Ps. fragi. The pasteurized cream included samples that had been allowed to stand at relatively low temperatures (5° to 10° C.) for several days and almost invariably a defect suggesting Ps. fragi was present. When plated on nile blue sulfate medium the cream was usually found to contain Ps. fragi in high numbers and frequently this organism was the predominant type.

Butter

The samples of butter examined were obtained from various creameries in Iowa and in surrounding states. A majority of the samples were abnormal in some respect and lipolytic cultures were isolated from a high percentage of these.

A large number of the samples of butter examined were definitely rancid or suggested rancidity. When plated many of them yielded cultures of Ps. fragi and it was noted especially that defective butter from one Iowa creamery almost invariably contained Ps. fragi in large numbers. Occasionally samples of rancid butter were received that on plating yielded comparatively few or no lipolytic organisms. In the case of some of these samples it was thought that the defect was due to growth of bacteria and that the lipolytic count had originally been high. However, due to the toxic products formed, the bacteria were rapidly killed off and by the time the butter was received for examination the numbers were relatively low. Probably with certain samples the defect was not of bacterial origin.

During an investigation of cheesy butter a number of lipolytic cultures were encountered. The defects in the cheesy butter ranged from a mild cheddar flavor to a very disagreeable putrid condition. The butter examined was plated with beef infusion agar plus 0.5 ml. of skim milk per plate for the purpose of picking proteolytic rather than lipolytic organisms. When the cultures isolated were inoculated into cream and the cream churned it was found that certain of them produced rancidity in the butter. Usually these cultures were gram negative rods of varying sizes and the majority were not active fat hydrolyzers. The lack of activity, so far as fat hydrolysis was concerned, probably accounted for the fact that the butter

was cheesy rather than rancid. In the work of Collins and Hammer (8) it was noted that in many instances organisms which are lipolytic do not produce rancidity in butter. This may be due to one of several reasons. Certain types appear to grow poorly or not at all in butter. Others may develop and, being proteolytic as well as lipolytic, bring about a cheesy condition rather than rancidity. Still other types appear to hydrolyze the fat and at first produce a definite rancidity but later bring about cheesiness; with these organisms it is conceivable that the fatty acids are used soon after they are freed.

Miscellaneous Dairy Products

In several instances cottage cheese was found to have undergone deterioration so that it was unfit for use. The defective material possessed an odor that strongly suggested the presence of Ps. fragi and when it was plated, using the nile blue sulfate technic, this organims was generally found in large numbers.

Two cultures of lipolytic bacteria were secured from a sample of rancid ice cream. Presumably the mix or some of its ingredients had become contaminated and allowed to spoil before freezing, because the finished ice cream had been kept properly hardened.

A piece of packing from a homogenizer was examined for lipolytic bacteria and several cultures were isolated. The

homogenizer had been used constantly for some time but had been kept in good condition. The isolation of lipolytic organisms from this material indicates the possibilities in connection with the contamination of dairy products from such equipment.

In plating a butter culture, a product that normally contains S. lactis and citric acid fermenting streptococci, a liquefying yeast was found to be present in relatively large numbers. The yeast hydrolyzed fat readily. While it would not be expected that lipolytic organisms would be encountered often in butter cultures, their occasional presence indicates the wide distribution of these types.

Other Materials

Samples of animal fat were obtained from the Iowa State College abattoir and held at room temperature in sterile containers for an extended period. No attempt was made to use aseptic procedures in removing the fat from the animals. When examined for fat hydrolyzing organisms several cultures were obtained from hog fat and one was isolated from sheep fat.

The plating of specimens of water from Lake LaVerne, a small lake on the Iowa State College Campus, yielded only a few cultures of lipolytic bacteria. Several attempts at isolation were made and it appeared that lipolytic organisms occurred rather infrequently in the water.

SECTION III. THE IDENTIFICATION AND CLASSIFICATION OF CERTAIN OF THE ORGANISMS ISOLATED

PART I. STUDIES ON *PSEUDOMONAS FRAGI*

The plating of various dairy products by the use of the nile blue sulfate technic frequently yielded cultures which, on comparatively young plates, possessed a sweet ester-like odor resembling that of the flower of the common Mayapple (Podophyllum peltatum). The ability of the cultures to hydrolyze cottonseed oil varied considerably, some being strongly lipolytic and others only weakly lipolytic. It was noted that the cultures were found most often in samples of various dairy products held at low temperatures over a period of time. A study of the cultural characters, biochemical features and growth conditions of this group of cultures led to the conclusion that it represented but a single species, although the reactions were not always identical. This variation in reactions occurred especially in the colony appearance and in the action on litmus milk. From apparently pure cultures sub cultures could be obtained that would give reactions markedly different from those of the original and it appeared that these cultures were variants of a central type.

Of a total of 58 cultures studied in detail 22 were obtained from raw milk, 11 from raw cream, 11 from pasteurized cream, 11 from butter, 2 from the water of Lake LaVerne and 1 from a sample

of sheep fat that had been held in the laboratory for a short time.

Identity of the Organism

The cultures studied are considered to be Pseudomonas fragi an organism which was investigated by Hussong (17). With reference to morphology, biochemical features and growth conditions the cultures obtained in the present study appeared to agree closely with those isolated by Hussong. One difference noted was in the ability to ferment arabinose. The production of acid but no gas from arabinose was found to be variable with the cultures isolated while those studied by Hussong consistently produced acid from this compound. In view of the fact that the organism is known to vary greatly in other respects, considerable variation in action on carbon materials would be expected.

General Description of Pseudomonas fragi

Morphology (Cultures grown at 21° C.)

Form and size. Rods; 0.5 to 1.0 by 0.75 to 4.0 microns when grown 1 day on beef infusion agar.

Arrangement. Singly, in pairs and chains of varying length. In 1 day cultures on beef infusion agar extremely long chains often noted.

Motility. Motile by means of a single polar flagellum.

Staining reaction. Gram negative.

Spores. Not produced.

Cultural Characteristics (Cultures grown at 21° C.)

Agar slope. On beef infusion agar after 1 to 2 days an abundant, spreading, raised, white and shiny growth. Some cultures of butyrous consistency, others viscid. Generally possessed a sweet ester-like odor resembling that of the flower of the Mayapple.

Agar stab. On beef infusion agar surface growth, with a smaller amount of growth extending along the line of inoculation.

Agar colony. On beef infusion agar colonies were generally irregularly rounded with entire edge, raised, smooth and from 4 to 6 mm. in diameter. Occasionally variant types noted that were thin and transparent or raised rough and thick. Some cultures viscid while others were not.

Gelatin stab. Crateriform to stratiform liquefaction in 3 to 4 days.

Beef extract broth. Turbidity and sediment.

Potato. Abundant, white to dirty white shiny growth.

Litmus milk. Action on litmus milk variable. Most freshly isolated cultures first formed a thin pellicle and then a ring of acid curd at the surface of the milk which was clearly evident after 5 days. At this time a Mayapple

odor was generally present. In some cases after approximately 10 days, acid formation was noted in the lower portion of the tube while in other cases it was not. After 2 to 3 weeks coagulation generally complete throughout the milk; coagulation apparently due in some cases to the formation of acid and in others to the elaboration of a sweet curdling enzyme. Usually milk was partially digested after several weeks incubation. With certain cultures no action observed on litmus milk beyond a slight pellicle formation.

Biochemical Features (Cultures grown at 21° C.)

Indol. Not produced.

Nitrates. Not reduced.

Hydrogen sulphide. Not produced in agar.

Methyl red reaction. Negative.

Voges Proskauer reaction. Negative.

Action on carbohydrates, etc. Dextrose and galactose fermented with the production of acid but no gas. Arabinose attacked with the production of acid but no gas by some strains and not attacked by others. Neither acid nor gas produced from glycerol, inulin, lactose, levulose, maltose, mannitol, raffinose, salicin and sucrose.

Hydrolysis of fat. Fat hydrolyzed.

Growth Conditions

Oxygen relationships. Aerobic.

Growth temperatures. Growth at 10° C. 30° C. and temperatures in between. No growth at 37° C.

Special Characters of Ps. fragi

Hydrolysis of Fat

When each of 44 cultures were spotted on plates containing cottonseed oil, 28 of them hydrolyzed only a part of the globules of fat below the growth while 16 hydrolyzed all of the globules. The cultures were relatively consistent in the production of a diffusible lipolytic enzyme, only one culture failing to hydrolyze the fat in a considerable area around the growth.

Hydrolysis of Simple Triglycerides

Tripropionin and tributyrin were hydrolyzed by all of the 42 cultures studied except a few that failed to grow on the media due, presumably, to the toxicity of the compounds freed. Trivalerin and triolein were hydrolyzed by a majority of the 42 cultures while triisovalerin, tricaproin, tricaprylin and tricaprin were hydrolyzed by some cultures but not by others. Triheptylin, trilaurin, trimyristin, tripalmitin and tristearin

were not attacked.

Action on Butter

Each of 10 cultures was inoculated into pasteurized cream and the cream churned. Immediately after churning counts on the unsalted butter ranged from 109,000 to 269,000 per ml. while after 4 days at room temperature the counts varied from 16,000,000 to 390,000,000 per ml. After 7 days at room temperature eight of the samples of butter had developed cheesiness while two showed a slight rancidity.

Variability of the Cultures

In the study of Ps. fragi it was noted that certain variations occurred more or less frequently. Generally the variations involved primarily the colony appearance and the action on litmus milk.

Hussong (17), in 1932, made an exhaustive study of variation in Ps.fragi and found that variation occurred in cultures purified by the ordinary methods and also in cultures purified by the single cell technic. Three distinct colony types were described by him. The S type was encountered most often in isolations from dairy products and was considered to be the normal type. It produced a smooth, glistening, convex, opaque colony on beef infusion agar. In litmus milk the S type first

produced an acid ring at the surface and later formed a coagulum at the surface along the wall of the tube. Later a more extensive coagulation of the milk occurred and a slight digestion of the curd followed. Fat was hydrolyzed. The O type produced a thin, translucent, smooth-edged and smooth surfaced colony. It produced little or no change in litmus milk beyond a slight reduction of the litmus in the bottom of the tube. Fat was generally not hydrolyzed. The R type produced colonies of various sizes and shapes. Some had a smooth surface and were translucent, with irregular edges. Others were large, very tough and wrinkled. The action of the R colonies on litmus milk differed only in degree from that of the S colonies. The action on fat was variable.

In the work herein reported no complete study was made of the variations occurring in Ps. fragi but the general results obtained confirmed the observations of Hussong. The majority of the cultures freshly isolated from dairy products were of the S type and possessed all of the characters of this type. It appeared that, when first isolated, the cultures were least inclined to vary, especially if carried through rather rapid transfers in litmus milk. When such cultures were plated the colonies were almost entirely of the S type. Variations in the species seemed to be induced by long holding at 5° to 10° C between transfers. When cultures that had been held at these temperatures for extended periods were plated, numerous R type

colonies were often found.

The type of colony did not appear to be a stable character. Starting with any one type it was possible to obtain both of the other types. The ability to hydrolyze cottonseed oil likewise was not a stable character. Certain cultures that were definitely lipolytic when first isolated, either failed to attack the fat or were weakly lipolytic after being held for an extended period.

Distribution of the Organism

While only 58 cultures of Ps. fragi were studied in detail, it should be noted that during the plating of a considerable number of normal and abnormal samples of milk, cream, butter, etc. many additional cultures were encountered. The products examined were obtained from Iowa State College and also from various other points in Iowa and in surrounding states. The organism could almost always be isolated from samples of raw milk and cream held at low temperatures over a period of time, indicating that the species is psychrophilic and rather regularly present in milk. Frequently samples of pasteurized cream were received that had undergone a change in flavor and odor while being held in refrigerators in the homes of consumers. The odor of the defective product often suggested the odor that is more or less characteristic of Ps. fragi and when such samples were plated this organism was generally found in large

numbers. Since Ps. fragi has been shown to be killed by relatively low temperatures (17), its presence in pasteurized milk probably indicates contamination after the heating.

In connection with studies on the spoilage of unsalted or low salted butter from an Iowa creamery, it was noted that the defect suggested Ps. fragi and on plating the butter this organism could almost always be found in large numbers. The investigation of a number of cases of deterioration in unsalted or low salted butter led to the conclusion that the causative organism was nearly always Ps. fragi. It should be emphasized that this species appears to be widely disseminated and it is entirely possible that it, more than any other organism, is responsible for defects in samples of cream and butter held at low temperatures.

PART II. STUDIES ON ACHROMOBACTER OLEIFINDENS (NOV. SP.)

Among the cultures encountered more or less frequently in the plating of various dairy products by use of the nile blue sulfate technic were a number that gradually brought about an acid reaction in litmus milk. A study of these cultures indicated they were closely related and belonged to the same species. It appeared that the organism was unrelated to any previously described species and the name Achromobacter oleifindens is proposed for it. The organism was not especially active in the

hydrolysis of fat as judged by the nile blue sulfate method, a majority of the cultures hydrolyzing only a part of the fat globules under the colonies. The species is of special interest in that, unlike most of the lipolytic microorganisms known, it produces acid and finally coagulates the milk without conspicuous digestion.

A study was made of 32 cultures of Ach. oleifindens isolated from various sources. Eighteen of these were obtained from raw milk supplied to Iowa State College by various producers, ten from raw mixed milk in the holding vat, three from raw sweet cream and one from homogenizer packing. The morphology, cultural characteristics and biochemical features of the organism were investigated and a description was prepared.

General Description of Achromobacter oleifindens (nov. sp.)

Morphology (Cultures grown at 21° C.)

Form and size. Rods; 0.4 to 1.0 by 0.4 to 12.0 microns when grown on beef infusion agar 1 to 2 days.

Arrangement. Singly, in pairs and chains.

Motility. Non-motile.

Spores. None produced.

Cultural Characteristics (Cultures grown at 21° C.)

Agar slope. Growth on beef infusion agar after 1 to 2 days was abundant, white, spreading, shiny and generally viscid.

Agar stab. Abundant surface growth with some growth following the line of inoculation in 1 to 2 days on beef infusion agar.

Agar colony. After 4 days on beef infusion agar surface colonies raised, white, generally viscid, convex, smooth, round, with entire to slightly irregular edge, and from 4 to 6 mm. in diameter. Subsurface colonies smaller, oval, white and generally viscid.

Gelatin stab. No liquefaction. Abundant development on surface of the stab with growth following the line of inoculation.

Beef extract broth. After 3 days cloudiness and some sediment. A pellicle formed by some strains but not by others.

Potato. A moderate, grayish white to buff colored growth evident after 4 days incubation.

Litmus milk. A pellicle generally formed after 4 days incubation. After approximately 9 days a definite reddening throughout the tube but no indication of coagulation. After 3 weeks the milk was both acid and coagulated and the litmus was generally slightly reduced at the bottom of the tube.

Biochemical Features (Cultures grown at 21° C.)

Indol. Not produced.

Nitrates. Not reduced.

Hydrogen sulphide. Not produced in agar.

Methyl red reaction. Negative.

Voges Proskauer reaction. Negative.

Action on carbohydrates, etc. Acid but no gas produced from arabinose, dextrose, galactose and lactose by some strains and not by others. Neither acid nor gas produced from glycerol, inulin, levulose, maltose, mannitol, raffinose, salicin and sucrose. Starch not hydrolyzed.

Hydrolysis of fat. Fat hydrolyzed.

Hemolysis. Red cells not hemolyzed.

Growth Conditions

Oxygen relationships. Aerobic.

Growth temperatures. Growth at 10°, 40° C. and temperatures in between. At 45° C. growth by certain strains but not by others.

Special Characters of Ach.oleifindens

Hydrolysis of Fat

The 32 cultures hydrolyzed cottonseed oil when tested by the nile blue sulfate method. Eleven of the cultures hydrolyzed all of the fat globules below the colonies while 21 hydrolyzed only a part of them. Twenty-seven of the cultures hydrolyzed the fat globules beyond the edge of the colony while five hydrolyzed only beneath the colony.

Hydrolysis of Simple Triglycerides

All of the 32 cultures hydrolyzed tripropionin, tributyrin, triisovalerin, triheptylin, tricaprylin and triolein while none hydrolyzed trimyristin, tripalmitin and tristearin. Action was variable on trivalerin, tricaproin, tricaprin and trilaurin.

Action on Cream

Each of 13 of the cultures was inoculated into sterile cream. After 9 days incubation at 21° C., seven cultures had developed a slight rancidity, four had developed a fecal odor, while the remaining two cultures developed only a slight off odor. After 16 days incubation at 21° C. the results obtained were essentially the same as after 9 days.

Action on Butter

Each of five of the cultures was inoculated into pasteurized cream and the cream churned. When the unsalted butter was held at room temperature (approximately 21° C.) none of the cultures brought about rancidity after 7 days. Three of the cultures produced a putrid odor while the remaining two did not produce any defect.

PART III. STUDIES ON ALCALIGENES LIPOLYTICUS

During the plating of a large number of samples of milk,

cream and various other materials for lipolytic organisms, using the nile blue sulfate technic, a species differing from the usual types of lipolytic organisms in that the colonies were very small in size was occasionally encountered. When picked into litmus milk it produced no change. An outstanding character of the organism was its pronounced lipolytic ability, as evidenced by the rapid hydrolysis of the fat globules under the colonies and by the extent of the zone of hydrolysis.

A study was made of 21 cultures of the organism. Fifteen of these were obtained at various intervals from the raw milk of one producer supplying Iowa State College and one came from the raw milk of another producer. Four cultures were isolated from raw mixed milk and another was obtained from the water of Lake La Verne.

Identity of the Organism

The organism isolated showed characters that indicated it belonged to the species isolated by Evans (12), in 1916, and designated Bacillus abortus var. lipolyticus. Evans found the organism produced a very faint growth in the form of small separate colonies on meat infusion agar slants. Gelatin was not liquefied, nitrates were not reduced and acid was not produced from carbohydrates. Growth was slight in litmus skim milk and the medium remained unchanged, while in litmus whole milk there

was good growth with slow acid development which was first apparent in the cream layer. Evans considered 37° C. the optimal growth temperature.

In a later paper Evans (13) pointed out that Bacillus abortus var. lipolyticus could be found in large numbers in milk and that it was killed by a temperature of 52° C. for 30 minutes or 63° C. for 30 seconds. In 1918, the same author (14) noted that this organism did not form endospores and accordingly belonged in the genus Bacterium. She suggested that the variety designation was unwieldy and that the organism was likely a distinct species. The name Bacterium lipolyticus was proposed. The evidence indicated Bact. lipolyticus was not pathogenic for guinea pigs.

A study of the morphology, cultural characteristics and biochemical features of the 21 cultures isolated showed that they were the same as the organism described by Evans (12). This study has permitted an extension of the original description. The characters of the species are such that it probably belongs in the genus Alcaligenes and the name Alcaligenes lipolyticus is proposed.

General Description of Alcaligenes lipolyticus

Morphology (Cultures grown at 21° C.)

Form and size. Rods; 0.6 to 1.0 by 1.0 to 1.4 microns when

grown 1 day on beef infusion agar. When grown approximately 3 weeks the cells became elongated measuring up to 0.8 microns in width and 2.4 microns in length.

Arrangement. Singly and in pairs.

Motility. Non-motile.

Staining reactions. Generally gram positive in young cultures. Older cultures gram negative.

Spores. None produced.

Cultural Characteristics (Cultures grown at 21° C.)

Agar slope. A scanty, white, filiform, non-viscid, dull growth on beef infusion agar after 1 to 2 days, the type of growth not changing on extended incubation.

Agar stab. Small amount of surface growth on beef infusion agar with growth extending down along the line of inoculation.

Agar colony. Growth evident on beef infusion agar in approximately 2 days while after 4 days surface colonies were white, non-viscid, round with entire edge and from 1 to 2 mm. in diameter. Subsurface colonies oval, white, non-viscid and smaller than surface colonies.

Gelatin stab. No liquefaction. A scanty growth on the surface of the gelatin and some growth following the line of inoculation.

Bouillon. A slight cloudiness in the medium and a slight sedimentation after 3 to 4 days.

Potato. No visible growth.

Litmus skim milk. Beyond a slight precipitate in the tube on extended incubation, no action observed in litmus skim milk.

Litmus whole milk. After approximately 2 weeks incubation acid apparent in the cream layer, the acid later extending down through the milk itself.

Biochemical Features (Cultures grown at 21° C.)

Indol. Not produced.

Nitrates. Not reduced.

Hydrogen sulphide. Not produced in agar.

Methyl red reaction. Negative.

Voges Proskauer reaction. Negative.

Action on carbohydrates, etc. In general neither acid nor gas produced from the compounds used. A few cultures produced acid but no gas from arabinose, dextrose, lactose, levulose and maltose. Galactose, glycerol, inulin, mannitol, raffinose, salicin and sucrose not fermented and starch not hydrolyzed.

Hydrolysis of fat. Fat hydrolyzed.

Hemolysis. Red cells not hemolyzed.

Growth Conditions

Oxygen relationships. Aerobic

Growth temperatures. All cultures grew at 10° C. at 37° C. and at temperatures in between. At 40° C. the majority of the cultures also gave slight growth.

Special Characters of Alc. lipolyticus

Hydrolysis of Fat

All of the 21 cultures hydrolyzed cottonseed oil when tested by the nile blue sulfate technic and they were relatively consistent in the type of lipolysis produced. Complete lipolysis beneath the growth occurred with all of the cultures while all except one hydrolyzed the fat for a considerable distance beyond the edge of the colony. The ability of the cultures to hydrolyze other fats was not tested as the work of Hammer and Collins (7) indicates that essentially the same results are secured with various natural fats.

Hydrolysis of Simple Triglycerides

All of the cultures hydrolyzed triisovalerin, tricaproin, tricaprylin, tricaprin, trilaurin and triolein. Tripropionin and tributyrin were hydrolyzed by a majority of the cultures; those not bringing about hydrolysis were unable to grow on the

media. Trivalerin, triheptylin, trimyristin, tripalmitin and tristearin were not hydrolyzed.

Action on Cream

Each of 21 cultures was inoculated into a small amount of sterile cream. After 7 days at 21° C. all of the cultures had developed rancidity, the defect being very pronounced.

Action on Butter

Each of 11 cultures was inoculated into pasteurized cream and the cream churned. The unsalted butter was stored at 21° C. After 3 days seven of the cultures had developed rancidity while after 5 days all of the samples were rancid.

Effect of Glycerol and the Sodium Salts of Fatty Acids on the Growth of Alc. lipolyticus

The fact that the group of otherwise relatively inert organisms attacked fat so readily suggested that they used one or more of the products of hydrolysis. This theory was tested by noting the effect of glycerol and of the sodium salts of various fatty acids on the growth of the organisms. Plain beef extract broth and beef infusion agar were used as controls; glycerol and the sodium salts in various concentration were added to other lots of broth and agar and the reactions of the

media were adjusted to pH 6.8. Each medium to be tested was inoculated with each of the 21 cultures. The results obtained after an incubation of approximately 1 week at 21° C. were as follows:

GLYCEROL.

Concentrations of 0.5 per cent of glycerol in the broth and agar did not influence the growth of the organisms.

SODIUM ACETATE.

In broth containing 0.5 per cent sodium acetate growth was much heavier than in the control tubes. Concentrations between 0.25 and 0.5 per cent in agar slants also increased the growth. In the control tubes the growth was thin, dull, beaded and almost streptococcus-like while in the slopes containing sodium acetate it was luxuriant, white and spreading.

SODIUM PROPIONATE.

Concentrations of 0.5 per cent of sodium propionate in broth greatly aided growth while the same concentration in agar completely inhibited development. When 0.25 per cent sodium propionate was added to the agar there was good growth but it was not quite as extensive, as in the agar to which sodium acetate had been added.

SODIUM BUTYRATE.

The addition of 0.5 per cent sodium butyrate to broth

gave the best growth in the series and with some cultures pellicle formation was noted. A 0.25 per cent concentration in agar gave a very good growth, as compared to that in the control, while 0.5 per cent inhibited growth.

SODIUM CAPROATE.

When 0.25 per cent sodium caproate was added to broth growth was greatly stimulated. The same concentration in beef infusion agar inhibited development while 0.1 per cent gave remarkably good growth.

SODIUM CAPRYLATE.

Sodium caprylate appeared to be extremely toxic and the concentrations used were necessarily very low; 0.1 per cent in broth and 0.05 per cent in agar brought about much better development than that in the control tubes.

SODIUM CAPRATE.

Sodium caprate was also relatively toxic, as compared to the other compounds used. Concentrations of 0.1 per cent in broth and 0.05 per cent in agar stimulated growth.

SODIUM OLEATE.

Growth of all the cultures was distinctly aided by the addition of 0.5 per cent sodium oleate to broth and 0.25 per cent to the agar.

Ability to Use Various Fat Components as the Sole Source of Carbon

The ability of Alc. lipolyticus to use glycerol and certain of the fatty acids as the sole source of carbon was investigated. The synthetic medium A of Ayres, Rupp and Johnson (2) was used as a control. It had the following composition:

Sodium ammonium phosphate	grams	2.0
Dextrose	"	10.0
Potassium chloride	"	0.1
Distilled water	ml.	1000.0

The test media were made up by varying the source of carbon in the above medium. Instead of dextrose the following compounds were used in the amounts designated: 0.25 per cent glycerol, 0.25 per cent sodium acetate, 0.25 per cent sodium propionate, 0.25 per cent sodium butyrate, 0.05 per cent sodium caproate, 0.05 per cent sodium caprylate, 0.05 per cent sodium caprate, and 0.1 per cent sodium oleate.

All of the cultures were able to grow to a slight extent in the control medium containing dextrose as the sole source of carbon. The organisms were also able to utilize glycerol and the sodium salts of the fatty acids designated as carbon sources but the various salts differed in their ability to support growth. The development in the media containing sodium acetate, sodium butyrate and sodium oleate was very good as compared to that in the control tubes while growth in the media containing glycerol, sodium propionate, sodium caproate, sodium

caprylate and sodium caprate was relatively poor.

Production of Acetylmethylcarbinol Plus Diacetyl in Skim Milk

Each of four cultures was inoculated into skim milk and the milk incubated at 21° C. for 3 days. At the end of this time the cultures were examined for acetylmethylcarbinol plus diacetyl. These compounds were not found.

Each of two cultures were inoculated into 3 portions of skim milk to which had been added 0.4, 0.5 and 0.6 per cent citric acid respectively. After 5 days incubation at 21° C. the cultures were examined for acetylmethylcarbinol plus diacetyl. None was found.

Production of Volatile Acid

The production of volatile acid was studied with three cultures by inoculating each of them into skim milk and incubating the milk 6 days at 21° C. When volatile acidity determinations were then made it was found that no increase had occurred.

Distribution of the Organism

The work of certain investigators has indicated that organisms which should be designated Alc. lipolyticus may

often be present in milk. Evans (12) studied the bacteria of milk freshly drawn from the normal udder and found Bacillus abortus var. lipolyticus in 33 or 12 per cent of the 192 samples examined. The organism was also present in the milk of all of the herds examined. In a later paper (13) the same author reported the isolation of Bacillus abortus var. lipolyticus from 9 of 23 milk samples investigated. Steck (23) in 1921 studied the bacteria present in the normal udder and reported the occurrence of organisms of the type described by Evans. Dorner (10) investigated the bacterial flora of aseptically drawn milk and found a large number of rods which he considered identical with the species isolated by Evans.

The present work indicates that Alc. lipolyticus is not disseminated as widely as Evans and others appeared to find. At one time the organism could be obtained with relative ease from the milk of one producer but numerous attempts to isolate it from the milk supplied by other producers and from various other materials generally resulted in failure. Later attempts to obtain the organism from the original sources likewise resulted in failure although repeated platings were made.

PART IV. STUDIES ON MYCOTORULA LIPOLYTICA

The plating of both normal and abnormal dairy products occasionally yielded yeast cultures that were characterized by

a lipolytic as well as a proteolytic ability. The various cultures appeared to belong to the same species although there were certain minor variations in colony structure and in the fermentation reactions. The fact that the organism was isolated from a number of samples of cheesy butter suggests its possible relationship to the defect.

Eleven cultures were studied in detail; these were selected from a considerably larger number of cultures that had been isolated. Seven of the cultures studied were obtained at various intervals from the raw milk supplied to Iowa State College. Three were isolated from plates poured for yeast and mold counts on samples of abnormal butter; two of the samples were slightly cheesy while a third was slightly rancid. One culture was found in an abnormal butter culture.

Identity of the Cultures

The cultures obtained were identified as Mycotorula lipolytica which was studied by Harrison (16), in 1928. Although Harrison did not record the action of the organism on fat, the other characters given by him agree closely with those of the cultures isolated. He reported that the cells were ellipsoidal to cylindrical, with occasional hyphal-like threads. On wort agar the growth was spreading, dull, rugose and whitish. Dextrose, mannose and glycerol were fermented with the production

of acid but no gas. Milk was almost completely peptonized and a thin white film appeared on the surface. Gelatin was completely liquefied in 24 days. The organism grew well at 25° C. but grew very little at 37° C.

The torula isolated by Rogers (21), in 1904, showed characters that relate it to M. lipolytica. It had a weakly lipolytic action that was not constant but appeared to vary with some unknown factor. Sugars were not fermented. At 30° C. milk was digested slowly with no previous coagulation. The organism developed readily under both aerobic and anaerobic conditions.

A detailed study was made of the morphology, cultural characters, and biochemical features of the 11 cultures and the description given the organism by Harrison (16) has been enlarged.

General Description of Mycotorula lipolytica

Morphology (Cultures grown at 21° C.)

Form and size. Ellipsoidal to cylindrical cells with buds at ends; 1.0 to 2.0 by 1.0 to 5.5 microns when grown 1 day on beef infusion agar. In older agar cultures and in subsurface colonies on plates, hyphal-like threads varying from 1.5 to 2.0 by 8.0 to 15.0 microns often observed.

Arrangement. Mature cells found singly; buds early broken from mother cell. In some instances relatively mature

cells found in pairs.

Motility. Non-motile.

Staining reactions. Gram positive.

Spores. None observed even in old cultures.

Cultural Characteristics (Cultures grown at 21° C.)

Agar slope. Abundant, flat to slightly raised, dull rough, spreading, white growth after 2 days on beef infusion agar, the type of growth not changing on extended incubation except to become slightly heavier. On wort agar essentially the same type of growth found.

Agar stab. On beef infusion agar after 1 to 2 days an abundant surface growth with relatively little or no growth following the line of inoculation. The surface growth was essentially the same in character as that on the agar slopes.

Agar colony. On beef infusion agar after 2 to 4 days surface colonies were white, dull, thin, rough, with uneven edge and from 2 to 6 mm. in diameter. Some surface colonies smaller, raised, convex, appearing almost like actinomyces colonies. Subsurface colonies generally smaller and having the appearance of mold colonies due to long cells growing out into the agar.

Gelatin stab. Stratiform liquefaction. Complete in 20 days or less.

Beef extract broth. After 4 days thin, white pellicle extending 5 to 8 mm. along side of tube above the surface of the liquid. Slightly turbid. Considerable sediment.

Potato. After 2 days a moderately heavy white to dirty white, dull growth. The type of growth was similar to that on agar slopes.

Litmus milk. After 3 days a thin white pellicle, with incomplete digestion throughout most of the tube. Occasionally a slight reduction of the litmus in the bottom of the tube. Digestion most pronounced on the surface of the milk. After 7 days, digestion complete, leaving brown to bluish brown serum. Pellicle generally became bluish white in color. Considerable sediment in the tube.

Biochemical Features (Cultures grown at 21° C.)

Indol. Not produced.

Nitrates. Not reduced.

Hydrogen sulphide. Not produced in agar.

Methyl red reactions. Negative.

Voges Proskauer reaction. Negative.

Action on carbohydrates etc. Acid but no gas in levulose by all cultures. Acid but no gas in dextrose and glycerol by some cultures but not by others. Neither acid nor gas produced from arabinose, dextrin, galactose, inulin, lactose, maltose,

mannitol, raffinose, salicin and sucrose.

Hydrolysis of fat. Fat hydrolyzed.

Growth Conditions

Oxygen relationships. Aerobic.

Growth temperatures. Growth at 10° and 21° C. At 37° C. growth occurred but was slight.

Special Characters of M. lipolytica

Hydrolysis of Fat

All of the cultures studied hydrolyzed fat actively. Eight of the eleven cultures hydrolyzed all of the globules below the growth while the remaining three hydrolyzed only a part of them. Seven hydrolyzed the fat around the colony growth while four did not.

Hydrolysis of Simple Triglycerides

All of the cultures hydrolyzed tripropionin, tributyrin, trivalerin, triisovalerin, tricaprylin, tricaprin and triolein; tricaproin and trilaurin were hydrolyzed slightly. Triheptylin was attacked by some cultures but not by others while trimyristin, tripalmitin and tristearin were not hydrolyzed.

Action in Cream

Each of the 11 cultures was inoculated into cream that had been sterilized in small flasks in approximately 100 ml. amounts. After 3 days at 21° C. all of the cultures had produced an off odor but none were typically rancid or cheesy. After 7 days, however, all of the samples of cream inoculated with the cultures were putrid, the defect being more pronounced with some cultures than with others. In this connection it should be noted that the organisms were actively proteolytic as well as lipolytic.

Action on Butter

Each of the 11 cultures was inoculated into pasteurized cream and the cream churned. After 1 day at 21° C., six samples of the butter were cheesy, three were cheesy to rancid while two were distinctly off but neither cheesy nor rancid. After 2 days at 21° C., however, 10 of the samples were cheesy and one was cheesy to musty. After 9 days at 5° to 10° C., three were cheesy to rancid and eight were cheesy while after 5 weeks four of the samples were cheesy, four were cheesy to rancid and three were definitely rancid. The counts on the butter held at 21° C. were not extremely high. After 2 days, at which time the butters were definitely cheesy, counts on six of the samples ranged from 2,200,000 to 27,000,000 per ml. The control sample had less than 100 organisms per ml.

Distribution of the Organism

For a number of years studies have been carried on at the Dairy Bacteriology Laboratories of the Iowa Agricultural Experiment Station on the organisms commonly found in dairy products. In the course of this work many cultures of liquefying yeasts have been obtained. While these were not tested for their ability to hydrolyze fat, their general cultural reactions indicated that they are the same as M. lipolytica. It should be noted that the ability to digest milk is not a common character among the yeasts found in dairy products and that the liquefying yeasts present in these materials appear to be closely related if not identical. M. lipolytica is characterized by its ability to bring about rapid and complete digestion of milk and its ability to grow vigorously on ordinary laboratory media. Cultures have been obtained from samples of milk, cream, butter and various other materials, indicating that the organism is widely distributed and present in a variety of products.

SUMMARY AND CONCLUSIONS

1. The nile blue sulfate technic, due to the inhibition of many non-lipolytic organisms, was found useful for the isolation of lipolytic types when they were present in small numbers as compared to the total numbers of organisms. However, it should be noted that certain lipolytic types, as well as the non-lipolytic ones, were also inhibited.

2. The nile blue sulfate technic gave low total bacterial counts due, presumably, to toxicity of the dye. When total and lipolytic counts were desired, the modified nile blue sulfate technic was very valuable. However, when the proportion of lipolytic organisms to total organisms was low the modified method was not especially useful. In addition the picking of lipolytic colonies was complicated by the flooding of the plates.

3. The simple triglyceride technic, employing either tripropionin or tributyrin, was not an accurate method for determining the ability of an organism to hydrolyze natural fats. S. lactis was found to hydrolyze tripropionin and tributyrin but did not attack either cottonseed oil or butter fat.

4. The natural fat technic was well adapted to the study of lipolysis because relatively high total and lipolytic counts could be obtained and the picking of lipolytic colonies was not complicated by flooding the plates. The primary disadvantage

of the method was the impossibility of detecting lipolytic organisms when they were present in small numbers, as compared to the total numbers.

5. Lipolytic bacteria were found distributed widely in both normal and abnormal samples of milk and cream. The abnormal samples included many that had been held at low temeratures for extended periods and these frequently contained Ps. fragi in large numbers.

The present of lipolytic organisms in pasteurized dairy products was considered to be due to contamination after heating since attempts to isolate lipolytic organisms from milk obtained from the vat immediately after pasteurization invariably resulted in failure although they had been shown to be present before heating.

The plating of samples of butter, most of which were abnormal in some respect, yielded many cultures of lipolytic microorganisms and certain of the samples contained Ps. fragi in large numbers. In addition to the materials already mentioned miscellaneous dairy products and various other materials yielded many cultures of lipolytic organisms.

6. The results obtained in a study of the Ps. fragi cultures isolated agreed with the conclusions of Hussong (17). Ps. fragi was found to be widely disseminated and was considered to be one of the organisms encountered most frequently in spoilage of certain dairy products. The spoilage in some cases was

a typical rancidity and in others it was an odor suggesting Ps. fragi.

7. A new lipolytic species, Achromobacter oleifindens, was described. The organism differed from the usual type of lipolytic culture because of the acid coagulation of litmus milk and of the failure to digest milk. The species was not strongly lipolytic and did not produce rancidity in butter.

8. A number of inert, lipolytic cultures were obtained and were considered identical with Bacillus abortus var. lipolyticus described by Evans (12). The characters of the organism indicated it belonged in the genus Alcaligenes and the name Alcaligenes lipolyticus was proposed. Alc. lipolyticus produced rancidity in butter and was characterized by its ability to rapidly hydrolyze fat and to use certain of the fatty acids as the sole source of carbon.

9. A yeast that was lipolytic, as well as proteolytic, was studied and found to be Mycotorula lipolytica, which was investigated by Harrison (16). The yeast attacked fat readily and some cultures produced rancidity in butter; others, however, brought about cheesiness. The organism grew well on ordinary laboratory media.

10. The results obtained on the numerous lipolytic cultures studied indicated that even such a character as the ability to hydrolyze fat may not be stable. The failure to hydrolyze fat was noted especially with variant cultures of

Ps. fragi.

11. Certain of the lipolytic cultures studied failed to produce rancidity in butter. The failure to produce this defect was thought to be due to poor growth or no growth in butter; in some cases when an organism was proteolytic as well as lipolytic a cheesy condition rather than rancidity resulted.

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