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PSEUDOMONAS GENUS

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HYDROLYSIS OF FATS BY BACTERIA OF THE *PSEUDOMONAS* GENUS^a

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It is well known that the deterioration of fats in foods may be caused by the action of certain bacteria. According to Jensen and Grettie (8) the rancidity produced by bacterial action most commonly involves two main types of chemical reactions, hydrolytic and oxidative. Flavor defects due to free fatty acids and oxidation products can be produced by single species capable of elaborating both hydrolytic and oxidative enzymes. This paper is devoted to the investigation of some of the factors involved in the hydrolysis of fats by members of the genus *Pseudomonas*.

Early studies on the action of microorganisms on fats have been reviewed by Jensen (7) and Lea (9). The work of Hammer and his colleagues (3, 6, 10) has shown that lipolytic bacteria of the *Pseudomonas-Achromobacter* groups are common in dairy products and cause flavor defects in butter.

Laboratory methods for studying the bacterial metabolism of fats have not, in general, been too satisfactory for obtaining consistent, reproducible quantitative data. Accurate analytical methods need to be devised for measuring the amounts of metabolic products formed. Procedures for emulsifying fats in a stable state of ultra-fine particle size have been lacking. Practically all of the early studies have been made using emulsified fats of variable, uncontrolled particle size. It has been demonstrated by Frazer and Walsh (4) that the rate of hydrolysis of a fat by lipase is proportional to the surface area and inversely proportional to the radii of the globules. In a very fine emulsion the reaction curve approximates that of a substance in true solution. One improved technique to increase the reactive surface of fat substrates was used by Trussell and Weed (11) who studied the lipolysis of staphylococci. These workers employed a culture shaking machine which kept the fat particles from coalescing and provided fresh surface for enzyme reaction. In the present study a further improvement in methods for producing stable, highly dispersed, fat emulsion media is reported.

MATERIALS AND METHODS

Media

Production of stable, finely-dispersed stock emulsions containing fats in concentrations as high as 25% is described below. From such stock emulsions it was convenient to make suitable dilutions with water to provide the desired experimental strengths in the media. The diluted emulsion-substrate was sterilized in Erlenmeyer flasks by auto-

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claving at 15 p.s.i.g. steam pressure for 15 minutes. A separately sterilized solution of supplementary nitrogenous and mineral nutrients was added aseptically to the sterile emulsion just prior to inoculation.

Preparation of emulsions. The technique for producing stable emulsions was a modification of the procedure described by Geyer, Mann, and Stare (5). The emulsifying agent used was a soy phospholipid (Astec 4135)^b obtained from the Associated Concentrates, Inc. Satisfactory emulsions were prepared from all of the animal and vegetable oils tested. The butter oil employed (Krafteen) was supplied by the Kraft Foods Co. Also tested were refined peanut, coconut, and soybean oils, as well as refined tallow and prime steam lard, all products being furnished by Armour and Co.

The details of a typical preparation are illustrated in the following example for producing 1000 ml. of an emulsion containing 20% butter oil. In a Waring blender, 20 g. of the emulsifying agent, Astec 4135, was dispersed in 100 ml. of water. To this agitated mixture 200 g. of butter oil, prewarmed to 60°C., was slowly added in small portions, thus providing a preliminary emulsification. After transfer to a larger vessel, the preliminary emulsion from the blender was cautiously diluted to 1000 ml. with warm water and heated, with continuous stirring, to 60°C. The final emulsification was conducted at 2500 p.s.i.g. in a dairy homogenizer (Cherry-Burrell Junior Viscolizer). Since the equipment was preheated with hot water, the emulsion temperature remained at approximately 60°C. during the homogenization. The product was continuously recycled and after 1 hour the emulsification was considered complete. Microscopic examination showed the oil globule diameters to be uniformly less than 0.5 micron. Emulsions so prepared do not break on autoclaving and the enormous oil surface exposed is readily available for bacterial attack. In general, concentrated emulsions containing approximately 20% fat were prepared in quantities of 3 to 6 liters, sterilized in 1 liter amounts, and stored under refrigeration until used. Such emulsions have remained stable for at least 3 months of storage.

Fat emulsion broths. Unless otherwise stated, lipolysis experiments were conducted using 300 ml. of emulsion broth per flask. To prepare 300 ml. of emulsion broth medium, the procedure adopted consisted of sterilizing in 500-ml. Erlenmeyer flasks 250-ml. portions of the emulsion suitably diluted from the stock emulsion. To this emulsion was added 50 ml. of a separately sterilized solution of supplemental nutrients adjusted to pH 6.8 with dilute hydrochloric acid. A preliminary fat analysis of the stock emulsion was made in order to prepare dilutions generally in the range of 4 to 6% fat for the final medium. The concentration of supplemental nutrients in the final medium, unless otherwise stated, consisted of 0.5% yeast extract and 0.1% each of primary and secondary potassium phosphates. Subsequent experiments have shown that secondary potassium phosphate in 0.2% concentration can be substituted for the mixture of primary and secondary phosphates.

Cultures

A variety of organisms were tested for their lipolytic power. In all, 25 cultures comprising members of the genera *Pseudomonas*, *Achromobacter*, *Alkaligenes*, *Candida*, and *Mycoderma* were given preliminary screening runs. One culture, *Pseudomonas fluorescens* (No. 52) was selected for detailed study in most of the experiments reported here. This culture, obtained from the collection of the Northern Regional Research Laboratory as NRRL-B12, was maintained in stock on agar slants (5% glycerol, 0.5% yeast extract, 1.8% agar) and inocula were prepared from actively growing subcultures in glycerol-yeast extract broth. The broth medium had the same composition as the solid medium except that the agar was omitted. Incubation was at 30°C. and the organisms were routinely subcultured daily for 3 or 4 days to obtain active transfers for lipolysis experiments. For the experimental runs the inoculum level employed was 3 ml. of an active 24-hour culture per 100 ml. of medium.

Isolation medium for lipolytic organisms. For detection and isolation of lipolytic organisms from food sources, an improved plating medium was devised. This plating medium, which is also suitable for estimating the numbers of lipolytic organisms in food samples, has been applied satisfactorily to such products as butter, pork trimmings, beef hamburger, dried peas, and beans. The medium consists of 1.5% butter oil in emulsion,

^b The emulsifying agent, Astec 4135, was resistant to bacterial hydrolysis and did not contribute to the free acidity formed in the lipolytic cultures.

0.5% yeast extract, 0.2% dipotassium phosphate, 0.005% Nile blue sulfate, and 1.5% agar.

The lipolytic agar medium was prepared by combining two sterile solutions, Mixture A and Mixture B, whose compositions are described as follows: For 1000 ml. of medium, Mixture A contained 15 g. agar, 5 g. yeast extract, and 2 g. K_2HPO_4 , which were dissolved in distilled water and made to a volume of 650 ml. The pH was adjusted to 7.2 with sodium hydroxide. Mixture B comprised 300 ml. of 5% butter oil emulsion plus 50 ml. of 0.1% Nile blue sulfate solution. The 5% oil emulsion used was derived by diluting a stock emulsion prepared as described above. After sterilization for 15 minutes at 15 p.s.i.g. steam pressure, the solutions (A and B) were cooled to 45° to 50°C., and combined under aseptic conditions. The final medium was thoroughly mixed before pouring into Petri plates.

To each plate was added 1 ml. of the sample dilution and 12 to 13 ml. of melted lipolytic agar medium, adequate care being taken to distribute the sample in the medium. Plates were incubated at 30-32°C. in a properly humidified incubator. Preliminary examination and colony count were made after 2 days of incubation, and a final count of lipolytic and non-lipolytic species was performed after 5 days of incubation.

The non-lipolytic colonies did not cause any change in the reddish hue of the slightly alkaline medium, and did not themselves turn blue. Lipolysis was characterized by a blue or blue-green color imparted to the individual colony and by a deeper blue zone developed in the medium immediately surrounding and underlying the colony. These zones gradually increased in size and became clearer and more transparent as incubation continued beyond the first few days.

Bacterial counts. The most probable number counts of bacteria in fat emulsion broth cultures were followed, using the procedure and probability tables of Buchanan and Fulmer (2). Serial dilutions were made of the culture, and each dilution was inoculated into 5 replicate tubes of glycerol-yeast extract broth which were then incubated at 30°C. for 72 hours.

Analytical

Samples of the culture liquor were removed aseptically by pipette for analysis.

Free fatty acid. A 10-ml. portion of culture liquor was heated with 30 ml. of isopropanol and 4 to 5 drops of phenolphthalein indicator (1% alcoholic) in a 60°C. water bath. Rapid titration with 0.100 *N.* aqueous sodium hydroxide gave sharp end points. Results are expressed as ml. of 0.100 *N.* acid per 10 ml. of culture liquor.

Extractable oil. The concentration of oil in culture liquors was determined by the following procedure: First, the emulsion was broken by a cadmium salt treatment and the mixture of fatty acids and oils was separated from the aqueous phase by extraction with an immiscible solvent. Then, the free fatty acids, converted to sodium salts, were separated from the lipid fraction. The washed oils, after evaporation of solvent, were dried to constant weight. The details of the determination follow. A 10-ml. sample of uninoculated emulsion broth or culture liquor was pipetted to a 50-ml. centrifuge tube and, after addition of powdered cadmium chloride sufficient to saturate the sample, the mixture was heated to approximately 65°C. in a water bath. The tube was permitted to stand at room temperature for 2 to 3 minutes and was then centrifuged for 1 to 2 minutes at 1,000 r.p.m. Since the clear supernatant layer of lipids could not be drawn off directly without appreciable losses, a volume of 5 ml. of benzene was added and thoroughly mixed with the contents of the tube. Separation of the benzene and aqueous layers was hastened by centrifuging the tube at 1,000 r.p.m. for approximately 1 minute and then drawing off the solvent layer using a pipette attached to a hypodermic syringe. It was found necessary to repeat this extraction process 10 times in order to extract all of the lipids present. The extracts, pooled in a separatory funnel, were repeatedly washed with small volumes (5 to 8 ml.) of aqueous 3% sodium carbonate solution to remove the free fatty acids from the glyceride fraction. When the washings no longer evidenced cloudiness, the free fatty acids were considered removed from the oils. The residual sodium carbonate was next removed by repeatedly washing the oil extract with warm water (55-60°C.) and the solution of glycerides and organic solvent was dried with anhydrous sodium sulfate. The drying agent was removed by filtering with suction through Whatman No. 5 paper and the solution, transferred to a tared weighing dish, was evaporated on a boiling water bath. The residual oil, dried to constant weight, thus represented the number of grams of glycerides present in 10 ml. of sample.

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Saponification. For anhydrous fats and oils the A. O. A. C. method (1) was used with a reflux period of 2 hours instead of the suggested 30-minute period. For determining the concentration of oil in an emulsion by the saponification method, the oil was first separated from the emulsion, as previously described for extractable oils, in order to remove the Astec emulsifying agent.

EXPERIMENTAL RESULTS

Effect of fat concentration on bacterial hydrolysis. Emulsion broth media containing butter oil concentrations varying in the range from 0.5% to 20% were inoculated with a representative lipolytic organism, *Ps. fluorescens*, No. 52. The media contained as supplements 0.5% yeast extract and 0.2% potassium phosphate. Incubation was at 30°C. and the flasks were shaken once daily. The rate of hydrolysis was followed by pH and titratable acidity measurements. The disappearance of fat from the emulsion cultures was indicated by the difference in extractable oil content before and after fermentation.

It will be seen from Figure 1 that the hydrolytic reaction at 30°C. is substantially complete at the end of 4 weeks. Plotted values are corrected acidities which represent the difference between the experimental flasks and

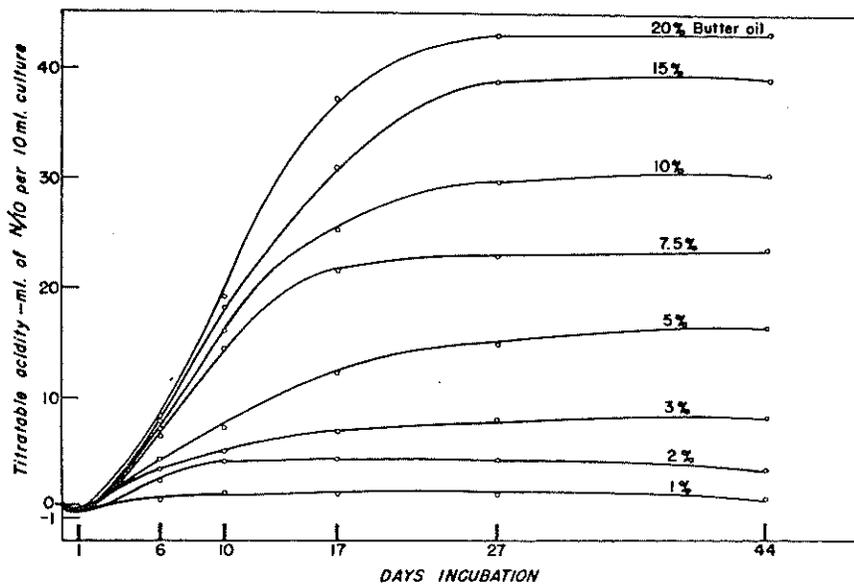


Figure 1. Rate of hydrolytic activity of *Ps. fluorescens* at different concentrations of butter oil.

their blanks. Control flasks are blanks consisting of uninoculated media which contained the same butter oil concentrations as these in the inoculated series. The blanks showed no significant change in acidity during the 44-day experimental period, thus indicating no hydrolysis of the oil emulsion in the absence of the lipolytic bacteria. Results are averaged from duplicate flasks. During the initial stage of incubation it has been regularly observed that a slight but definite decrease in acidity occurred before lipolysis was manifested. The dip in the acidity curve took place within the first

24 hours. Where little or no fat was present, this reduction in titratable acidity was more marked than at fat concentrations above 1%. It is presumed that the explanation for the preliminary drop in acidity is associated with the protein breakdown during the early growth of the bacterial cells. It was noted that when yeast extract, in the range varying from 0.1 to 10%, was added to emulsions with the same initial fat level, the 24-hour acidity drop increased with increasing concentrations of the nitrogenous nutrient, and the subsequent course of fermentation at protein levels above 1% yeast extract suggested that bacterial proteolysis was competing with and predominating over lipolysis.

As shown in Table 1, more than 95% of the fats were decomposed when the concentration of lipids in the emulsion media approximated the range

TABLE 1
Effect of fat concentration on hydrolysis of butter oil by *Ps. fluorescens*

Degree of hydrolysis		
Fat level in medium		Efficiency of hydrolysis ^c
Initial (0 days)	Final (44 days)	
<i>g. per 100 ml.</i>	<i>g. per 100 ml.</i>	%
20.0	7.23	63.9
10.0	1.20	88.0
7.5	0.36	95.2
5.0	0.13	97.4
1.0	0.03	96.6

$$^c \% = \frac{(\text{Initial fat concentration} - \text{Final fat concentration})}{\text{Initial fat concentration}} \times 100$$

between 1 and 8 g. per 100 ml. At higher fat levels somewhat lower lipolytic efficiencies were obtained. For the highest fat concentration tested, namely, 20 g. per 100 ml., the degree of lipolysis amounted to 63.9% of the fat originally present. The deviation from a linear relationship is apparent in Figure 2 where the degree of lipolysis, after 4 weeks of fermentation, is plotted as a function of the initial oil concentration.

Bacterial hydrolysis of various animal and vegetable fats. It has long been known that the physical properties of fats influence the degree of dispersion of oil-in-water emulsions, and that it is difficult to produce finely divided, stable emulsions with some of the higher molecular weight solid fats. If two fats of relatively different composition (butterfat and tallow, for example) can be maintained in the same state of subdivision, a comparison of the bacterial cleavage of these fats is then justified. It is suggested that the emulsification methods used by earlier investigators of bacterial lipolysis did not produce the same degree of dispersity essential for valid comparison. By neglecting these differences in dispersion, some authors concluded that the fats containing higher molecular weight fatty acids are more resistant to bacterial cleavage than those of lower molecular weight. Using emulsification procedures now available, however, the preceding concept can be re-evaluated. The experimental production of ultra-fine dispersions of fats makes possible comparisons where globule size is not a variable.

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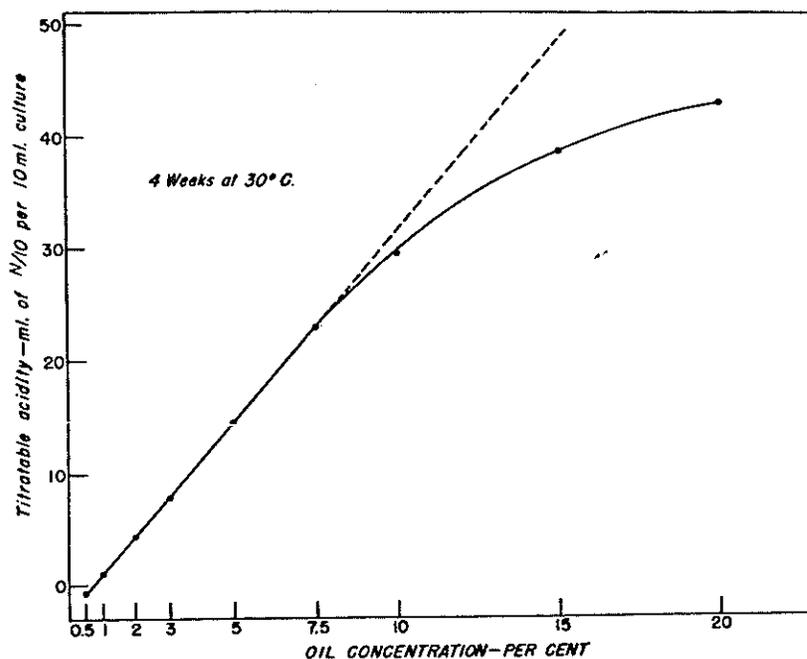


Figure 2. Effect of butter oil concentration on degree of hydrolysis by *Ps. fluorescens*.

In the present investigation a limited number of animal fats and vegetable oils were emulsified by the homogenizer technique described. Emulsion media containing yeast extract and potassium phosphate as supplements were seeded with active cultures of *Ps. fluorescens*, No. 52. The experimental results, shown in Table 2 and Figure 3, demonstrate that all of the fats tested were highly susceptible to microbial cleavage. Furthermore, the rate of hydrolysis followed the same general pattern for each of the different animal and vegetable fats and the high lipolytic efficiency shown for each of the oils was approximately the same.

Lipolytic activity of different *Pseudomonas* species. Fat-splitting enzymes have been reported in a variety of bacterial species. These include

TABLE 2
Hydrolytic action of *Ps. fluorescens* on different fats

Fat substrate	Saponification value	Degree of hydrolysis		
		Fat level in medium		Efficiency of hydrolysis ^d
		Initial (0 days)	Final (42 days)	
		<i>g. per 100 ml.</i>	<i>g. per 100 ml.</i>	%
Coconut oil.....	245	6.00	1.15	80.8
Butter oil.....	222	5.81	0.99	83.0
Tallow.....	189	6.05	0.96	84.1
Lard.....	188	7.11	1.83	74.3
Soybean oil.....	184	6.64	1.63	75.5
Peanut oil.....	183	6.25	1.31	79.0

^d See footnote to Table 1.

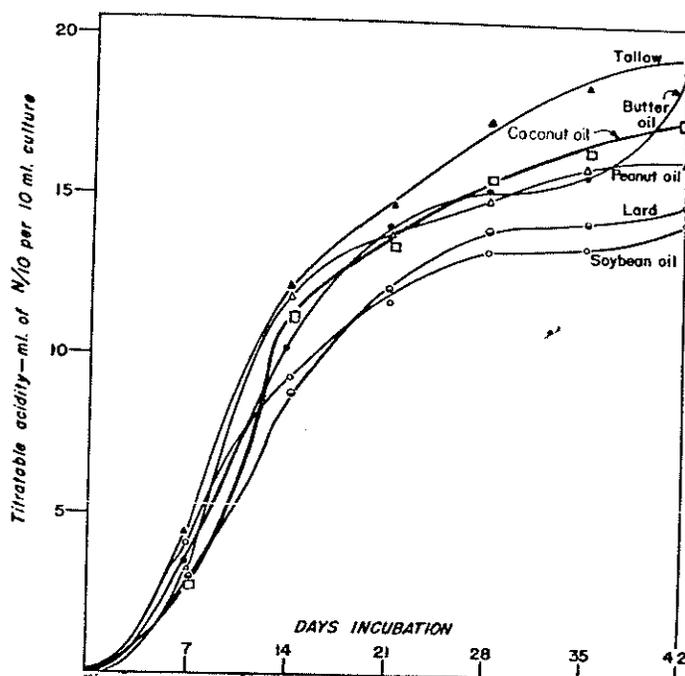


Figure 3. Hydrolysis of animal and vegetable fats by *Ps. fluorescens*.

members of the following genera: *Micrococcus*, *Bacillus*, *Escherichia*, *Corynebacterium*, *Pseudomonas*, and *Achromobacter* (7). In the present investigation the lipolytic activity of 8 members of the *Pseudomonas* genus was determined.

Emulsion broth media containing 5.48% butter oil, yeast extract, and potassium phosphate were inoculated with actively growing cultures of the species indicated in Table 3. At intervals during the incubation at 30°C. data were collected for titratable acidity values and pH. In addition, bacterial cell counts and extractable oil analyses were made on 4 of these cultures, as shown in Tables 3 and 4.

Wide differences in lipoclastic activity were revealed among the individual *Pseudomonas* species and between strains of the same species as shown in Table 3. Four of the cultures, Numbers 52, 57, 58, and 60, were strongly hydrolytic. Attention is called to the slow rate at which *Ps. oleovorans*, No. 60, proceeded during the first 4 weeks of incubation, and the rapid action during the next fortnight. In contrast to the dip in the acidity curve during the first day shown by *Ps. fluorescens*, there was a continuous decrease in titer for 21 days before the inflection point was reached in the case of *Ps. oleovorans*. Cultures No. 59 and No. 114 may be grouped together as organisms of only moderate lipolytic power. Based on 6-week acidity curves, Culture No. 99, *Ps. ovalis*, was classified as non-lipolytic, and Culture No. 100, *Ps. jaegeri*, was considered weakly lipolytic.

Commonly used tests for fat splitting are based on an increase in acidity after relatively short incubation periods. Slow-fermenting bacteria may be erroneously classified as non-lipolytic under the conditions of such tests.

TABLE 3
Hydrolytic activity of different *Pseudomonas* species on butter oil

Culture No.	Degree of hydrolysis		Efficiency of hydrolysis ^a	Rate of hydrolysis												
	Fat level in medium			Titratable acidity (corrected) [†] (ML. of 0.1 N. per 10 ml. of Culture)	Days of Incubation at 30°C.											
	Initial	Final			0	2	5	7	12	14	21	28	34	42		
	<i>g.</i> per 100 ml.	<i>g.</i> per 100 ml.														
52	<i>Ps. fluorescens</i>	5.48	0.51	90.7	0	0.1	0.9	7.5	11.9	11.4	15.0	16.9	17.2	18.8		
57	<i>Ps. fragi</i>	5.48	1.36	75.2	0	0	1.0	2.4	5.1	6.0	9.2	11.5	13.2	15.7		
99	<i>Ps. ovalis</i>	5.48	5.02	8.4	0	-0.5	-0.7	-0.8	-1.4	-1.5	-1.6	-1.7	-1.7	-1.9		
100	<i>Ps. jaegeri</i>	5.48	3.74	31.7	0	-1.1	-0.4	-0.4	-0.4	-0.1	0.7	1.4	2.3	3.1		
58	<i>Ps. fragi</i>	0	0.7	0.9	2.9	5.8	6.7	12.1	14.3	15.0	16.2		
59	<i>Ps. fragi</i>	0	0.4	1.4	1.9	2.9	3.9	5.8	7.6	9.9	11.9		
60	<i>Ps. oleovorans</i>	0	-0.2	-0.7	-1.0	-1.5	-1.6	-1.8	-0.5	10.6	17.7		
114	<i>Pseudomonas sp.</i> (From sweet cream)	0	0.1	0.2	0.4	1.1	1.7	4.2	6.0	7.0	8.3		

^a See footnote to Table 1.

[†] Corrected acidity equals acidity of inoculated medium minus acidity of uninoculated medium.

TABLE 4
Bacterial growth rate in butter oil emulsion[‡]

Days of Incubation	Millions of Bacteria per ml. of Culture			
	No. 52 <i>Ps. fluorescens</i>	No. 57 <i>Ps. fragi</i>	No. 99 <i>Ps. ovalis</i>	No. 100 <i>Ps. jaegeri</i>
0.....	350	110	25	350
2.....	2,500	1,300	800	3,500
5.....	7,000	2,500	1,100	25,000
7.....	1,700	2,500	1,300	18,000
12.....	2,500	2,500	800	5,000
14.....	1,300	800	500	2,500
21.....	35	35	350	500
28.....	5	13	500	800
34.....	0.35	0.01	130	700
42.....	0.001	0.00025	35	1,000

[‡] Butter oil concentration, 5.48%; 30°C. incubation.

Failure to consider the incubation time factor and the occasional low acidities which fall below the initial level (negative acidity) may result in erroneous conclusions in interpreting such data. In the instance of *Ps. jaegeri*, extractable oil values showed that 31.7% of the fat had disappeared (a substantial degree of lipolysis) although acidity figures were relatively low. If a reaction time of 4 weeks were arbitrarily chosen, both *Ps. oleovorans* and *Ps. ovalis* would have fallen in the same acidity classification and have been described as non-lipolytic. At 6 weeks incubation, however, *Ps. oleovorans* is grouped with the active lipolytic cultures. It has been postulated in the preceding discussion that negative acidity values result when lipolysis is masked by competing, concurrent reactions associated with proteolysis. Thus, titratable acidity figures serve as a reliable index of fat-splitting ability only when the cultures are moderately to strongly active and when the time of incubation is sufficiently prolonged.

Changes in the bacterial population of Culture Numbers 52, 57, 99, and 100 are shown in Table 4. Illustrated in Figures 4, 5, and 6 are rate of hydrolysis and bacterial growth curves. The initial lag phase of growth is not plotted, since 2 days elapsed in each case before the second count was made. In the 4 cultures studied the bacterial population rose sharply and attained a maximum in approximately 5 days. As the acidity increased, the number of viable cells declined. In the actively lipolytic cultures the pH dropped from an initial value of 6.8 to levels of 5.0 to 6.0 in 21 days, at which time the bacterial count had fallen to a point below the initial count made at the time of inoculation. At the end of 42 days a further drop in pH, reaching 4.5 to 5.0 in the strongly lipolyzing cultures, coincided with a drastic reduction in cell count. It is of interest to note that hydrolytic activity was prolonged in Cultures No. 52 and 57 despite the fact that the bacterial growth was reduced to extremely small numbers. Additional work is planned to further explore the mechanism involved.

SUMMARY

An improved procedure for studying the bacterial metabolism of fats has been developed. It includes improvement in the preparation of stable fat emulsion media and in methods of measuring bacterial cleavage. The

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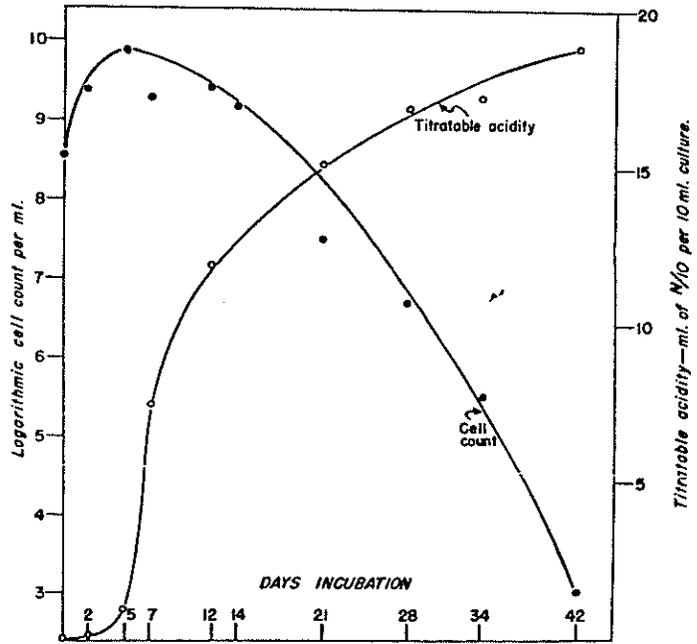


Figure 4. Rates of hydrolysis and bacterial growth in butter oil emulsion cultures of *Ps. fluorescens*, No. 52.

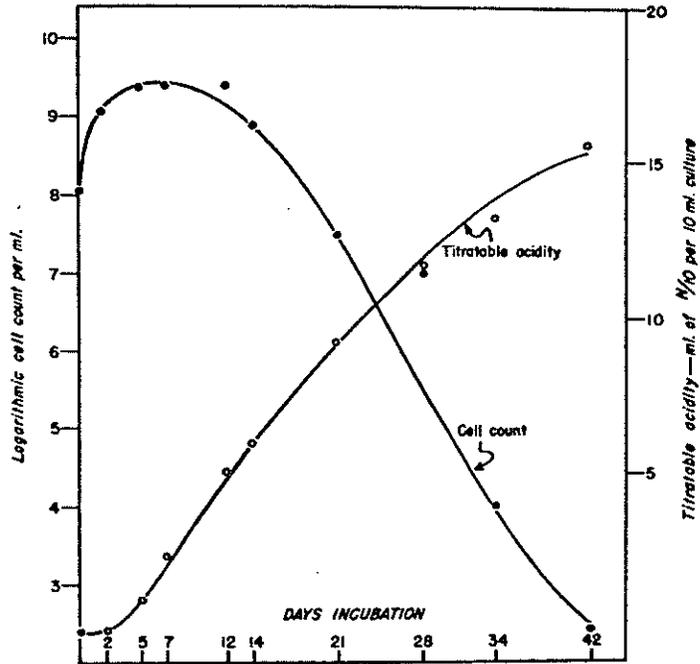


Figure 5. Rates of hydrolysis and bacterial growth in butter oil emulsion cultures of *Ps. fragi*, No. 57.

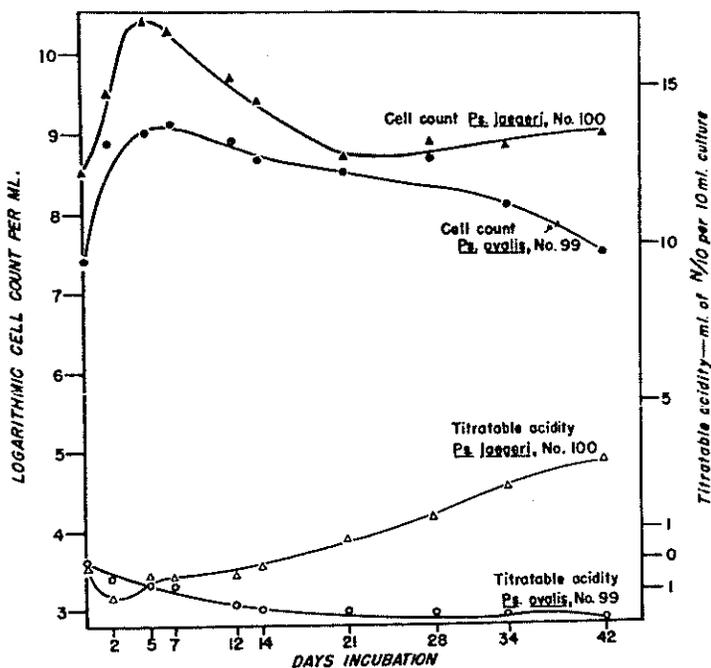


Figure 6. Rates of hydrolysis and bacterial growth in butter oil emulsion cultures of *Ps. ovalis*, No. 99, and *Ps. jaegeri*, No. 100.

importance of having finely-divided globules available in order to provide a highly effective surface for bacterial action has been demonstrated for a variety of animal and vegetable fats. Susceptibility of different fats to microbial hydrolysis appears dependent upon the particle size of the emulsified fats and not on the molecular composition of the component triglycerides. Thus, substantially the same degree of cleavage was achieved with fats possessing widely varying fatty acid compositions as butter, lard, tallow, coconut, soybean, and peanut oils.

Emulsified fats, subjected to attack by *Pseudomonas fluorescens*, undergo hydrolysis to a degree which is practically complete (90-95%) at fat concentrations below 10 g. per 100 ml. of medium. At higher fat levels up to 20 g. per 100 ml. the extent of hydrolysis deviates below the 90-95% value found in the dilute emulsions, the deviation being greater with increasing fat concentrations.

The composition of the culture medium markedly influences the type of reaction particularly for microorganisms which possess active proteolytic as well as lipolytic enzymes. When fats were supplemented by low protein levels, the *Pseudomonas* test organisms reacted mainly in a lipolytic manner. In contrast, proteolytic competition with lipolysis was indicated by lower acidity titers when larger amounts of nitrogenous supplements were added to the emulsion media.

Lipolytic powers of different strains and species of the *Pseudomonas* genus were found to vary widely in both rate and extent of hydrolysis. Of the 8 cultures tested, *Ps. fluorescens*, *Ps. oleovorans*, and 2 strains of *Ps.*

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fragi were strongly lipoclastic. Another strain of *Ps. fragi* and an unidentified species of *Pseudomonas*, No. 114, isolated from cream, possessed moderate fat-splitting ability. Weak activity was shown by *Ps. jaegeri* and none was exhibited by *Ps. ovalis*.

Acknowledgment

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