

LIPOLYTIC ACTIVITY OF MICROORGANISMS AT LOW AND INTERMEDIATE TEMPERATURES. I. ACTION OF *PSEUDOMONAS FLUORESCENS* ON LARD*

JOHN A. ALFORD AND L. E. ELLIOTT^b

Meat Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland

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The importance of lipolytic activity of microorganisms in meats, meat products, and dairy products held at low temperatures has been recognized for many years (5, 6, 7, 9). Although there have been numerous investigations on various factors affecting the production of lipase by microorganisms, only a few have been concerned with the effects of temperature. Nashif and Nelson (10) found that *Pseudomonas fragi* had an optimum temperature around 15° C for lipase production and that several other Gram-negative bacteria produced maximum quantities of lipase around 20° C even though the optimum temperature for activity of the lipases was near 40° C (12). Greene and Jezeski (5) reported a close correlation between total bacterial counts and onset of lipolytic activity by a *Pseudomonas* species over a temperature range of 0° to 30° C.

Balls, Matlack, and Tucker (1), in studies with pancreatic lipase, found that the hydrolysis of the higher, but not the lower, saturated triglycerides was dependent upon temperature. When the pancreatic lipase was allowed to act for 72 hours at 0° C, the percentage hydrolysis of each triglyceride was as follows: C₄, 57%; C₆, 75%; C₈, 95%; C₁₄, 4%; C₁₆, 2%; C₁₈, 3%. However, at 40° C for 3 hours, the percentage hydrolysis of each triglyceride was: C₄, 60%; C₆, 76%; C₈, 96%; C₁₄, 99%; C₁₆, 97%; C₁₈, 87%.

In the above microbial investigations in which the effect of temperature was studied (5, 11), the substrates were coconut oil and butterfat. Both of these lipids contain appreciable amounts of the short-chained fatty acids. The fat of pork and beef contains no short-chained fatty acids; yet their deterioration at low temperatures is of considerable economic importance. An investigation was initiated, therefore, to study the effect of temperature on the lipolytic activities of psychrophilic bacteria on animal fats. For such an investigation it was necessary to determine the best method for preparation of emulsions and to consider temperature and other factors involved. This paper is a report on the methods of study and the results of the action of a strain of *Pseudomonas fluorescens* on lard.

EXPERIMENTAL METHODS

The lard used was rendered in an open kettle according to standard practice without the addition of an antioxidant. It was packaged in 1-lb vacuum-packed cans and stored at -30° C until used in emulsions. Several relatively simple methods for preparing emulsions were tried. These were emulsification in a hand homogenizer with various

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^b Deceased.

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emulsifying agents and the use of a Waring blender or Omni-Mixer^b for the emulsification of lard-emulsifier mixtures. None of these procedures gave stable emulsions for prolonged periods of storage. The most satisfactory stock emulsions were prepared in a manner similar to that described by Goldman and Rayman (4). In this procedure, preliminary emulsions of the lard (20% concentration), emulsifier (Astec 4135^c, 2% concentration), and water were made at 57° C ($\pm 3^\circ$ C) in a Waring blender and then homogenized in a Manton-Gaulin homogenizer. A 2-liter dispensing funnel served as a reservoir on the intake side of the homogenizer and the liquid from the discharge side flowed into it to permit recycling of the emulsion. The reservoir was covered and a stream of nitrogen was continuously introduced into this closed, recirculating system. The temperature of the liquid was maintained at 55° to 60° C during homogenization by directing a stream of cold water over the homogenizer block and discharge pipe. The emulsion was recycled for 25 min at a pressure of 3500 psig. After homogenization it was placed in screw-capped bottles and autoclaved at 121° C for 15 min. When sterilization was completed, nitrogen was introduced into the autoclave as the pressure dropped, thus filling the head space of the bottles with nitrogen. The caps were screwed on tightly and the bottles stored at 3° to 5° C until used. Nearly all of the globules in these emulsions were less than one micron in diameter, and the emulsions were stable for several months. Peroxide values on these emulsions ranged from 1 to 7 and remained practically unchanged during storage.

The bacterium used in this investigation, *Pseudomonas fluorescens*, ATCC 11251, had been carried in stock culture for several years. This culture was moderately lipolytic and would grow at a temperature as low as -4° C.

In one series of experiments, the fat was present in the growth medium (initial pH 7.0) and the titratable acidity was determined at intervals from this culture. Uninoculated flasks incubated under identical conditions served as controls and the titratable acidity of the controls was subtracted from those of the inoculated flasks to give the net acidities shown in the data.

In the other series, the culture was grown in 1% peptone broth containing 0.05 M phosphate (initial pH 7.0 except in trials involving effect of pH) and the lipolytic activity of the culture supernatant determined at intervals. The supernatant was assayed as follows: Ten ml of supernatant containing the enzyme was added to a flask containing 5.0 ml of a 20% lard emulsion, 10.0 ml of 0.25 M phosphate buffer (0.25 M KH_2PO_4 adjusted to pH 7.0 with 0.25 M Na_2HPO_4), 1.0 ml of 1.8% formaldehyde (final concentration of 1:3000) and water to make a total volume of 50 ml. These flasks were incubated at 30° C for 24 hr. Assay medium was adjusted to pH 7.0 on the basis of preliminary work which showed that the optimum pH for activity of the enzyme ranged from 6.5 to 7.5. A similar activity range had been shown for lipase from other species of *Pseudomonas* (10), although Lubert, Smith, and Thornton (8) found an optimum pH of 8-9 for a strain of *P. fluorescens*. Control flasks containing equivalent amounts of supernatant heated to 90°-98° C for 10 min were prepared in a similar manner. The titratable acidities of the control flasks were subtracted from those of the inoculated flasks and the differences in these acidities at zero and 24 hr are the net values shown in the data. It was found that the difference in the zero-hour and 24-hr titratable acidities of the inoculated flasks gave a net acidity very close to that in which the heated control flask was taken into account, and it could have been used without affecting the results.

The titratable acidity of all samples was determined as follows: A 10-g aliquot was weighed into a Mojonnier fat-extraction flask and acidified with 25% H_2SO_4 to approximately pH 2.5. Ten ml of ethyl alcohol were added and the mixture shaken thoroughly. Samples from flasks in which the organisms were grown in the presence of lard were extracted three times with 10 ml of petroleum ether. Samples from flasks containing culture supernatant and buffered lard substrate were extracted twice with 20 ml of a 1:1 mixture of ethyl ether and petroleum ether. Extracts were titrated with 0.02 N alcoholic NaOH using phenolphthalein as the indicator.

^b The mention of specific trade names throughout this paper does not constitute endorsement of the product used over comparable equipment and materials.

^c Produced by American Lecithin Company, Inc., 57-01 32nd Avenue, Woodside, Long Island 77, New York.

Four media were compared to determine which would give maximum lipase production when lard in a final concentration of 5% was added. These media were: 1% peptone broth containing 0.05 M phosphate as suggested by Nashif and Nelson (10), Goldman and Rayman's medium (4) in which yeast extract was the carbon and nitrogen source, a mineral salts-phosphate medium (2) in which ammonium sulfate was the nitrogen source, and BBL Trypticase Soy Broth. These media were inoculated with a 24-hr suspension of cells washed from a veal infusion agar slant.

To determine the effect of pH on production of the lipase, peptone broth without fat was prepared and inoculated as described above except that the initial pH was adjusted as desired. The pH in one experiment was maintained within 0.2 pH units of the initial value by adding after each determination the amount of acid necessary to return the flask contents to the desired pH.

All cultures and flasks except aerated cultures were incubated in thermostatically controlled water baths with a sensitivity of $\pm 0.2^\circ \text{C}$. Aerated cultures were incubated in a room held at 20°C ($\pm 1.0^\circ \text{C}$) on a reciprocating shaker operating at 180 cycles per min with a stroke of $2\frac{1}{2}$ inches.

RESULTS

Results of the comparison of the 4 media for lipase production when lard was included in the growth medium are shown in Figure 1. The cultures grown in peptone-lard broth exhibited slightly higher activity than those in the Goldman-Rayman medium, although the difference was negligible for the first 10 days. This pattern of slightly greater lipolytic activity in peptone broth was repeated in cultures grown at 5°C and at 15°C . An additional experiment was carried out in which the culture was grown in these media without fat and the culture supernatant assayed for lipase activity. A similar slight superiority for the peptone broth was found in this comparison. On the basis of these findings the peptone broth was used throughout the remainder of the experiments.

The effect of pH on the production of lipase in a lard-free medium at 20°C is shown in Table 1. A similar series with the culture grown at 5°C is shown in Table 2. These data indicate that the optimum pH for production of the lipase is around 7.0, although there apparently was a broader optimum pH range at the lower temperature.

Nashif and Nelson (11) found more lipase activity in flask cultures of *P. fragi* than in cultures in test tubes; the differences in lipolytic activity, however, were less than the differences in total counts. Table 3 indicates that with the strain of *P. fluorescens* used

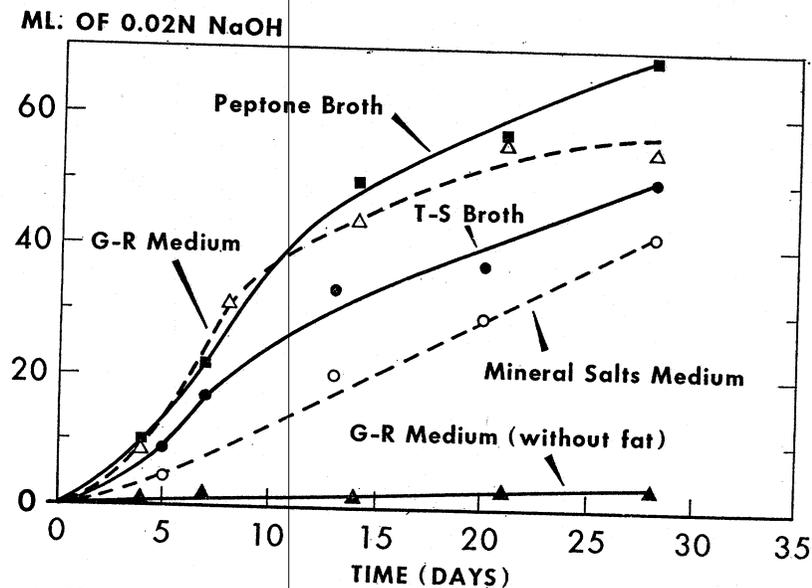


Figure 1. Lipase production at 20°C by *P. fluorescens* in various media containing lard.

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TABLE 1
Effect of pH on production of lipase at 20° C by *Pseudomonas fluorescens*

pH range ¹ or initial pH	pH and lipolytic activity of supernatant after:			
	3 days		4 days	
	pH	T. A. ²	pH	T. A.
6.0 to 6.2.....		0.2		0.5
6.0.....	6.3	0.3	6.6	1.0
6.5 to 6.7.....		3.4		3.8
6.5.....	7.0	3.4	7.3	4.7
7.0 to 7.2.....		5.1		5.5
7.0.....	7.6	4.4	7.8	5.3
7.5 to 7.7.....		4.5		5.1
7.5.....	8.0	2.7	8.2	4.3

¹ The pH range shows the maximum change in pH during incubation.
² Titratable acidity in Ml, 0.02N NaOH.

TABLE 2
Effect of initial pH on the production of lipase at 5° C by *Pseudomonas fluorescens*

Initial pH	pH and lipolytic activity of supernatant after:					
	7 days		14 days		22 days	
	pH	T. A. ¹	pH	T. A.	pH	T. A.
6.5.....	6.6	0.2	6.8	3.7	7.1	3.9
7.0.....	7.1	0.0	7.5	3.0	7.8	3.9
7.5.....	7.7	0.0	7.9	1.9	8.2	3.3

¹ Titratable acidity in Ml, 0.02N NaOH.

TABLE 3
Effect of surface-volume ratio on lipase production and total count at 20° C

Volume of medium ¹	Net activity after 3 days ²	Log of count after 3 days
50 ml.....	7.8	8.3
100 ml.....	2.2	7.9
200 ml.....	0.3	7.4
75 ml on shaker.....	2.0 ³	9.0 ³

¹ In a 300-ml Erlenmeyer flask, except 500-ml flask on shaker.
² Ml, 0.02N NaOH.

³ These values were attained in 24 hr.

in this investigation the surface-volume ratio affects lipase production in some manner other than merely an effect on total count. Aeration is not the only variable involved since cultures grown on a shaker had high counts but low lipase production. Similar results were obtained in experiments in which the culture was grown with fat in the medium and the titratable acidity determined on aliquots removed at intervals.

Figure 2 shows the effect of temperature on the total count, lipase production, and lipase activity in a lard-peptone medium incubated at different temperatures. It is apparent from these data that the optimum temperature for lipase production is definitely lower than that for growth when either rate or total numbers are used as criteria for the latter. Further evidence that this effect was an actual decrease in production of enzyme rather than an accelerated destruction at the higher temperature is shown in Figure 3 for cells grown in fat-free medium. Cells grown at 31° C produced very little lipase during the entire incubation period whereas appreciable quantities were produced at 5° and 20° C.

Table 4 shows that the optimum temperature for activity of the enzyme is apparently unaffected by the temperature at which the enzyme was produced. Since incubation at 30° C had been selected for the first assays in this study to conform with the temperature used by other workers (8, 10), the use of this temperature was continued even though greater activity occurred at 40° C.

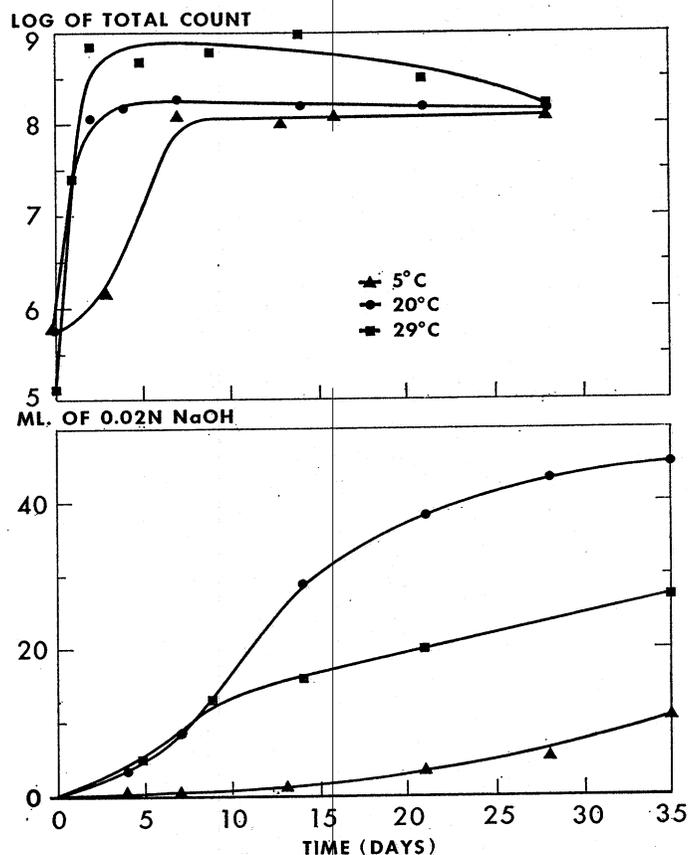


Figure 2. Effect of temperature on total count and lipolytic activity in lard-peptone medium.

TABLE 4
Effect of temperature on activity of lipase produced at different temperatures

Cultural conditions	Lipolytic activity ¹ of culture supernatant at:				
	20° C	30° C	40° C	46° C	50° C
5 weeks at 1° C	1.2	2.7	4.6	4.0	
3 days at 20° C	2.1	3.4	5.8	5.1	3.2
2 days at 28° C	1.0	1.5	2.5	2.4	

¹ Ml, 0.02N NaOH.

A comparison of the 5° C curves and the 20° C curves in Figure 3 indicates that there was no depressing effect of temperature on lipase production at low temperatures as was shown for the higher temperatures. Once the lag period in lipase production was passed, the amount of lipase for a given number of cells grown at 5° C was at least equal to that for a similar count on the 20° C culture.

DISCUSSION

The effect of medium composition on lipase production substantiates the findings of others (11, 13) that microorganisms produce lipase in the absence of lipids and that an easily metabolized carbohydrate source retards lipase

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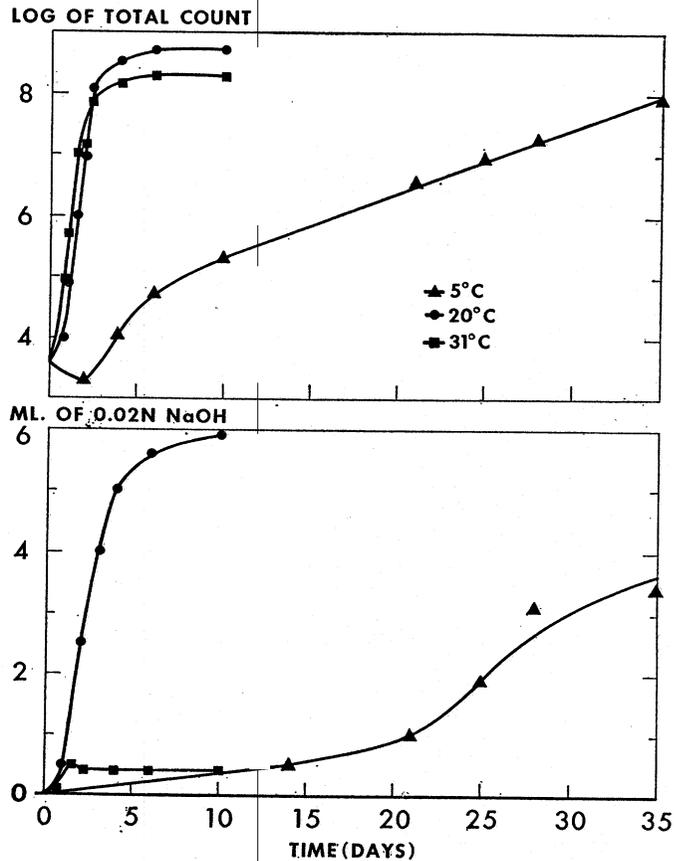


Figure 3. Effect of temperature on total count and lipase production in fat-free medium.

production. What might be interpreted as stimulation of lipase production by a simple nitrogen source probably should be interpreted as a non-specific response to a nutritionally inadequate medium. Because of the high yield of cells obtained with aerated cultures, this method of cultivation is often employed to give increased enzyme production. The data presented here, however, indicate that aeration retards production of lipase, although its production cannot be considered an anaerobic process since maximum yields were obtained in shallow, stationary layers of medium.

The depressing effect of increasing temperatures on the production of certain enzymes was noted several years ago by Gale (3), who found that the production of amino acid decarboxylases by *Escherichia coli* was inhibited by raising the growth temperature. However, since the decarboxylases were strictly adaptive, it is of interest that a similar effect may occur with a constitutive enzyme such as lipase. Additional studies have been made on the effect of elevated incubation temperatures on the reactions obtained in several tests used for the differentiation of microorganisms. These results are being reported elsewhere.

Data in Tables 1 and 2 suggest that the lipase produced by cells grown at low temperatures may have a slightly lower pH optimum than that of cells grown at higher temperatures. More evidence is necessary to show this to be a significant difference, but it does indicate that an active lipase can be produced at low temperatures in acid foods.

The observation that the quantity of lipase produced per cell at low temperatures is at least as great as that produced at temperatures nearer the optimum for growth of the bacterium or activity of the enzyme has important implications in the storage of foods. Bacteria growing on food products held at low temperatures may not cause apparent fat deterioration as long as the food is held near 0° C; short periods of elevated temperature, however, could bring about definite lipolysis by the enzyme which had been formed at the lower temperature.

Although it may be reasonable to assume that any change in lipolytic activity with temperature is a quantitative rather than a qualitative response, the effect of triglyceride chain length shown by Balls, Matlack, and Tucker (1) may also be involved. This point is now being investigated. In any case, the data reported here indicate that it is possible to use lard, when it is properly emulsified, as a substrate in lipolytic investigations, even though the incubation temperature is low.

SUMMARY

A study has been made of the effect of medium composition surface-to-volume ratio, pH, and temperature on the production and activity of lipase by *Pseudomonas fluorescens* when lard was the substrate.

It was found that 1% peptone broth with an initial pH of 7.0 gave maximum lipase production. The yield of lipase per cell was much greater in shallow, stationary layers than in deeper layers, or in aerated cultures. The optimum pH for production of the enzyme was about 7.0, although it was produced down to pH 6.0.

At 20° C easily detectable levels of lipase were observed in 2-3 days, whereas production was very slight at 30° C, even though good cell yields were obtained. The amount of enzyme produced per cell at 5° C was equal to that produced at 20° C. The optimum temperature for the lipase was near 40° C and was not affected by the temperature at which the cells were grown.

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