inversion cycles, and after 0.5 hour, creaming was negligible (<0.05 ml.). It also passed the emulsion-stability test as set forth in the military specification (*2*).

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MICROBIOLOGICAL FAT PRODUCTION

Effect of Fermentation Variables on Rate of Fat Formation by *Rhodotorula gracilis*

MARVIN P. STEINBERG and Z. JOHN ORDAL

Department of Food Technology, University of Illinois, Urbana, Ill.

A study was made of the effect of experimental variables on the rate of fat formation as distinguished from cell growth. Shake flasks and a fermentor assembly which included an automatic pH recorder-controller were employed. The fat content was expressed as a ratio of fat to nonfat yeast; this ratio increased linearly with time of fattening. The rate of fat formation varied linearly with pH between pH 3.0 and 8.5 and increased from 2.1 to 3.1 grams of fat per 100 grams of nonfat yeast per hour. Decreasing the temperature from 28° to 22° C. lowered the fat rate to less than half the original value. Under the experimental conditions employed, the addition of accessory growth factors, or of the cations calcium, sodium, and iron, to the growth medium appeared to be unnecessary for fattening of the yeast. Neither sugar concentrations up to 16% by weight of medium nor type of hexose had a significant effect on fattening. Acetate was inhibitory at pH 5 and was of no value as an adjunct at pH 8. Ethyl alcohol and glycerol did not give increased fat formation.

THE RATE OF FAT FORMATION of a variety of microorganisms has been investigated (*9*). Enebo *et al.* (*2, 9*) studied fat production by various *Rhodotorula*. They chose *Rh. gracilis* for detailed study because this yeast grew readily in submerged culture, it formed fat rapidly and abundantly, and the cells were easily removed from the culture medium. They demonstrated that when a medium containing soluble nitrogen and a relatively high concentration of sugar is inoculated, two cultural phases can be observed. During the first or "protein" phase the cells proliferate at a normal logarithmic rate and have a low fat content. In the second or "fattening" phase, which begins when the medium has been depleted of nitrogen, the cell population remains nearly constant but the fat content increases. These findings were confirmed by Pan *et al.* (*10*).

In these, as in most other investigations on fat formation by microorganisms, multiplication and fattening of the cells were carried out successively under the same set of conditions. Thus the investigator allowed the culture to grow under a given set of experimental conditions and then, without change in conditions, to form fat. No logical basis could be found for assuming that optimum conditions for multiplication would likewise be the optimum for fat formation.

If the nonfat portion of the yeast cell is considered as a factory for fat production, then, under a given set of conditions, fat should be produced at a constant rate. The specific rate of fattening for a given weight of yeast would depend on experimental conditions. This concept is supported by the data presented in this paper. The objective of this investigation was to determine the effect of various experimental conditions on the rate of fat formation by *Rh. gracilis* when the fattening phase was separated from that of growth or multiplication. Should fat production by this organism become commercially desirable, the rate of fat formation would be an important consideration.

Materials and Methods

Equipment A fermentor was used in the studies on pH and temperature; a shake flask technique was employed in the case of all other variables. The fermentor assembly, shown in Figure 1, included an automatic pH recorder-controller in addition to the usual air sparger, constant temperature water bath, and stirrer. The unbaffled stainless steel fermentation tank was 28.5 cm. in both diameter and depth.

The pH control equipment consisted of an immersion-type stainless steel electrode holder carrying Beckman industrial size electrodes (shown removed from the fermentation vessel), an amplifier, a controller, and a diaphragm-type pump. When the recorded pH deviated from the set point, an external electric circuit was closed, causing the pump to admit neutralizing solution from a reservoir. Because in these experiments the deviation was always toward the acid side, 1*N* sodium hydroxide was the only solution required. The total variation in pH

was ±0.2 unit from the set point.

Compressed air was continuously led through a centrifugal air cleaner, a diaphragm pressure-reducing valve, an orifice-type flowmeter, a cotton filter, and a sintered-glass air diffuser into the bottom of the fermentation tank. A downward thrust impeller, situated at an angle off-center and just above the air diffuser, was used for agitation of the liquid and further subdivision and dispersion of the air stream. For intermediate sampling of the cell suspension, a tube was led from the fermentation vessel to a 500-ml. suction flask.

For work with shake flasks, 1-liter Erlenmeyer flasks, each containing 225 ml. of inoculated medium and closed with a cotton pad, were placed on a shaking machine. The shaker was of the reciprocating type and was regulated to give a stroke of 3 inches at a rate of 100 excursions per minute. Heating elements and a cotton duck enclosure provided temperature control when the room was below 28° C.

Rhodotorula gracilis NRRL Y-1091 (obtained through the courtesy of L. J. Wickerham, Northern Regional Research Laboratory, Peoria, Ill.), a transplant of the strain used by Enebo *et al.* (*2*), was the yeast culture used in this study.

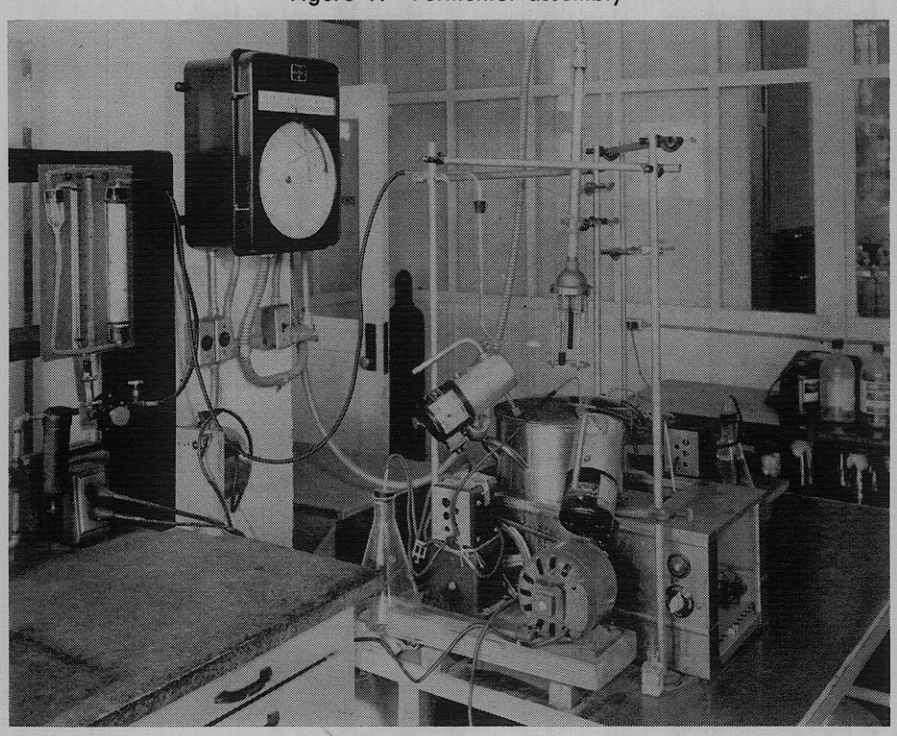
Cultivation The medium used for the production of yeast cells was a slight modification of that used by Enebo *et al.* (*2*). It included 20 grams per liter of corn sugar (Cerelose kindly supplied by the Corn Products Refining Co., Argo, Ill.), 3.0 grams of yeast extract (Difco), and 2.0 grams of ammonium sulfate. The corn sugar was separately sterilized and aseptically added to the balance of the medium just before use. The yeast for fattening was produced in shake flasks.

Fattening Prior to fattening in fermentor experiments, additional growth equivalent to approximately two generations was produced in the fermentor. One liter of yeast suspension, obtained from four shake flasks, and 9 liters of water were added to the fermentor together with 80 grams of corn sugar, 8 grams of ammonium sulfate, 12.0 grams of yeast extract, and corresponding amounts of salts. The pH controller was set at pH 5.0, the rate of aeration was adjusted to 0.5 liter of air per liter of medium per minute, and the thermostat was set for 28° C. Foaming of the medium was controlled by the dropwise addition of a silicone emulsion antifoam agent. This supplemental growth phase was continued for approximately 9 hours, then the pH or temperature control was set for the desired value and 375 grams of corn sugar were added to the medium. This point was taken as the beginning, or zero time, for the fat-forming process, which was continued for 36 to 40 hours. Periodically, simultaneous samples were taken in duplicate for determination of dry weight and fat.

Table I. Increase in Fat Content and Nonfat Yeast Concentration with Time at pH 4.5

Time, Hours	Dry Yeast, G./10 Ml.	Fat, G./200 Ml.	Fat Content		Nonfat Dry Yeast, G./L.
			%	Fat ratio	
0.0	0.0261	0.0262	5.00	0.053	2.48
12.0	0.0576	0.1914	16.6	0.200	4.81
24.0	0.0854	0.5431	31.8	0.467	5.82
38.0	0.1104	0.9987	45.2	0.825	6.05

Figure 1. Fermentor assembly



To prepare for fattening in shake flasks, the full-grown yeast culture was centrifuged and resuspended in distilled water of twice the original culture volume. Unless otherwise noted, the yeast harvest was not washed. After the pH had been adjusted to 7.2 ±0.2, the well-mixed slurry was dispensed into 1-liter Erlenmeyer flasks. The flasks were treated in duplicate as to experimental variable and placed on the shaker at 28° C. for about 36 hours. A single sample for dry weight determination and another for fat determination were taken from each flask, thus giving duplicate determinations for each condition.

Analytical Procedures The concentration of yeast in a suspension was determined as the grams of dry weight present in a 10-ml. sample. Centrifuged and washed cells were dried in a hot air oven at 105° C. for approximately 12 hours.

A 200-ml. sample of culture medium was taken for fat determination. To the yeast paste collected by centrifuging was added 25 ml. of 1*N* hydrochloric acid, and the mixture was placed in a steam autoclave for 15 minutes at 15 pounds per square inch pressure. After cooling, 10 ml. of water and 25 ml. of ethyl alcohol were added. The fat was extracted with three 50-ml. portions of Skellysolve F.

Results and Discussion

Expression Of Results The results from experiments on microbial fat production have commonly been reported as the per cent fat attained after a specified incubation period. Examination of the experimental results presented in Figure 3 shows that the time at which the experiment is terminated may greatly influence the conclusions reached. Furthermore, the percentage basis is a nonlinear one. Simple calculations show that the nonfat portion of the yeast cell must produce almost 2.5 times as much fat in going from 40 to 50% fat as from 10 to 20% fat.

It was therefore decided to express the the fat content as a ratio between the weight of fat and the weight of nonfat yeast solids. This expression has been designated as "fat ratio." Thus,

$$\% \text{ fat} = \frac{\text{grams of fat}}{\text{grams of dry yeast}} \times 100$$

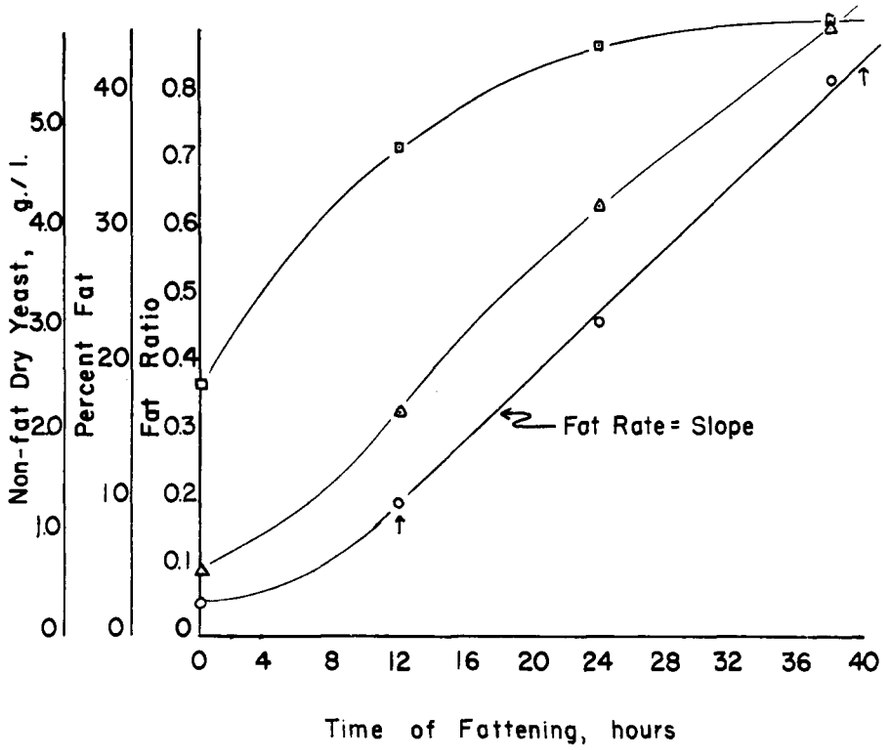


Figure 2. Increase of fat content and nonfat dry yeast with time at pH 4.5

○ Fat ratio △ % fat □ Nonfat dry yeast

and

$$\text{Fat ratio} = \frac{\%\ \text{fat}}{100 - \%\ \text{fat}}$$

It may be reasoned that a given quantity of cell protoplasm should produce an equal quantity of fat during any equal increment of time. If this were true, a plot of fat ratio against time should be a straight line whose slope is a rate. This has been termed the "fat rate," which is expressed as grams of fat per 100 grams of nonfat dry yeast per hour. Furthermore, this fat rate would be expected to vary with the environmental fattening conditions. That such was the case is shown below.

Effect of pH The literature was thoroughly examined for information in regard to the optimum pH for fat formation by microorganisms. Other than to state that fat formation requires an approximately neutral medium, no generalizations could be drawn.

The effect of pH on lipide production by *Rh. gracilis* was investigated through the range of pH 3.0 to 8.5 at intervals of 1.5 pH units. The data obtained from the experiment in which the pH was controlled at 4.5 during the fattening phase are given in Table I, columns 2 and 3. The fat content and the nonfat dry yeast concentration calculated from the data for each sampling time are also shown and are presented in Figure 2.

The concentration of the nonfat portion of the yeast almost doubled during the first 12 hours of fattening and thereafter approached a constant value. Similar plots for the other pH values investigated presented much the same picture. This high degree of separation obtained from the multiplication phase was considered sufficient to meet the objective for this investigation.

Following an initial lag period, the curve of fat ratio plotted against time in Figure 2 is a straight line. This fat rate is 2.40 grams of fat per 100 grams of nonfat dry yeast per hour. The fat ratio curves for pH 3.0 and 8.5 were chosen for presentation in Figure 3 because these levels represented the extremes used in these experiments. They show that although a longer lag was incurred at pH 8.5, this level gave a more rapid increase in fat content. This increase ceased before a fat content of 50% was attained, therefore higher pH values were not investigated.

A plot of fat rate against pH is given in Figure 4, in which each experiment is represented by a single point. The fat rate increased linearly with an increase in pH. This finding is probably of physiological significance and may be due to the effect of pH on acetate metabolism by the cell. Thus, Kempe *et al.* (*6*) and Halvorson (*4*) have shown that *Lactobacillus delbrueckii* shifts from the production of lactic acid to acetic acid as the fermentation pH is increased. Eoff *et al.* (*3*) found that *Saccharomyces ellipsoideus* formed more glycerol from sugar in alkaline than in acid solutions.

Kleinzeller (*8*), working with *Torulopsis lipofera*, found that the lipide content varied inversely with the pH of the medium. However, in that experiment he used a fattening time of only 3 hours and dealt with fat contents in the neighborhood of 5%. On the basis of the results found with regard to the increased lag caused at the higher pH levels, as shown in Figure 3, it may be presumed that Kleinzeller was working entirely within the intermediate phase. Therefore, his work corroborated the finding that raising the pH lengthens time taken before the true fattening phase sets in.

Temperature In order to obtain information on the effect of temperature on the rate of fat production, experiments were carried out at three temperatures—22°, 28°, and 35° C.—in the fermentor at pH 7.5. Inspection of the data in Table II reveals that the maximum fat rate occurred at 28° C. As reported by Enebo *et al.* (*2*), this is the optimum growth temperature for this organism. Based in part on his work with *Torulopsis lipofera* (*8*), Kleinzeller (*7*) states that this coincidence of optimum temperatures for both growth and fattening generally holds true in fat formation from carbohydrate by microorganisms.

The reduction of the temperature to 22° C. reduced the fat rate to less than one half that obtained at 28° C., indicating that control of temperature is of even greater importance in fat production than the control of pH.

Accessory Growth Factors Yeast extract was the sole source of accessory growth factors used in the growth medium. It was of interest to determine whether constituents of the yeast extract complex were of benefit to the fat-forming process as differentiated from that of growth.

Table II. Effect of Temperature on Rate of Fattening at pH 7.5

Temperature, °C.	Fat Rate[a]
22	1.09
28	2.79
35	2.68

[a] G. fat per 100 g. nonfat dry yeast per hour.

Table III. Fat Formation by Washed Cells (B) as Compared to Control (A)

	21.5 Hours		39.0 Hours	
	A	B	A	B
Fat ratio, g. fat g. nonfat dry yeast	0.51	0.55	0.85	0.93
Nonfat dry yeast concn., g./l.	6.34	5.10	6.54	5.13

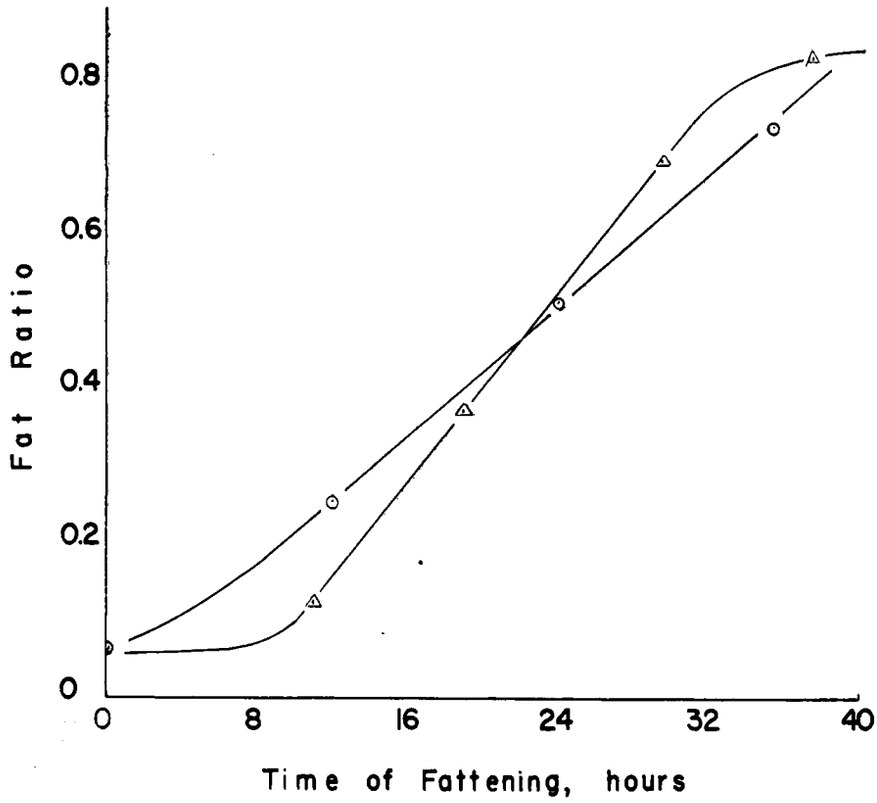


Figure 3. Variation of fat ratio with time at pH 3.0 and 8.5

○ pH 3.0 △ pH 8.5

The first experiment in this series was designed to determine whether any of the medium constituents not absorbed by the cell during growth were necessary in fat formation. Fattening of washed cells was compared to fat formation by cells remaining in the growth medium. From the results presented in Table III, it may be seen that the washed cells showed a slightly higher fat content at both sampling times.

As yeasts have the ability to store excess vitamins during growth, a cell crop was produced in a medium from which the yeast extract was omitted. Although the cell yield was somewhat reduced, normal fattening occurred. This investigation was then carried one step further in that the inoculum for cell production was also produced on media free from yeast extract—i.e., a double transfer. Cells produced under such conditions also synthesized fat (Table IV). This indicates that if additional accessory factors are necessary for fat synthesis, the requirements are of a very low magnitude.

Cells produced in the yeast extract—free medium had a very high fat content (22% at zero fattening time). Apparently the lack of accessory growth factors limited growth, yielding in the growth medium an excess of carbohydrate which was utilized for fat formation.

Sodium, Calcium, And Iron Enebo *et al.* (*2*) and Pan *et al.* (*10*) added sodium, calcium, and ferric chlorides to the medium used in their experiments. In order to determine whether or not these salts are needed for fat formation, the yeast was grown for two transfers in a medium containing no added sodium, calcium, or iron. The yeast extract was also eliminated. These cells also produced fat and gave results similar to those shown in Table IV. This would suggest that the amounts of these elements found in unrefined carbohydrates are sufficient to meet the requirements for fat synthesis by *Rh. gracilis*.

Sugar Concentration Enebo *et al.* (*2*) found that an 8% invert sugar solution could be used for fattening. Pan *et al.* (*10*) confirmed this finding. It was therefore decided to fatten yeast in media whose sugar concentrations ranged from 4 to 40% by weight. A plot of the results of this experiment, given in Figure 5, indicated that increasing the sugar concentration up to approximately 20% had no effect on the fat content of the cells. Above this concentration the fat content decreased. The samples at 40% sugar had a translucent appearance and showed no sedimented cells after the usual centrifugation.

Type of Sugar There is considerable controversy in the literature in regard to the best sugar substrate for fat synthesis. Limiting consideration to *Rh. gracilis* and the sugars glucose, fructose, and sucrose, Lundin (*9*) considered glucose most satisfactory, while Husain and Hardin (*5*) concluded that fructose was superior to either gluclose or sucrose. Pan *et al.* (*10*) found glucose and molasses equally satisfactory.

Glucose, fructose, and an equal mixture of glucose and fructose were compared as substrates for fat synthesis (Table V). Under the conditions of the experiment nearly equal amounts of fat were formed from each of the three substrates. The slightly higher fat ratio found for cells fattened on glucose is probably not significant. These data suggest that carbohydrates such as starch, sucrose, or inulin would, after proper hydrolysis, be of equal value as substrates for fat synthesis by this yeast.

Calculations based on the experimental data presented by Husain and Hardin (*5*) demonstrated that glucose gave a yield of 4.5 grams of nonfat yeast per liter, whereas with fructose as the substrate the yield was only 2.0 grams per liter. When fructose and glucose were used as a mixed substrate, an increase in the fructose-glucose ratio resulted in a decreased yield of nonfat yeast. This indicates that under their conditions fructose retarded the growth and multiplication of *Rh. gracilis* and explains their finding of a higher fat content in cells

Table IV. Effect on Subsequent Fat Formation of Double Transfer in Medium Free from Accessory Growth Factor

	Time of Fattening, Hours		
	0	21.5	42.5
Dry yeast, g./10 ml.	0.0634	0.1134	0.1457
Fat, g./200 ml.	0.2787	0.9405	1.3871
Fat content, %	22.0	41.4	47.6
Fat ratio	0.28	0.71	0.91
Nonfat dry yeast, g./l.	4.94	6.65	7.63

Table V. Evaluation of Several Substrates for Fat Synthesis

Substrate	Dry Weight, G./10 Ml. 23.5 hr.	38.0 hr.	Fat Ratio at 38.0 Hr.
Glucose	0.0935	0.1098	1.09
Fructose	0.0903	0.1089	1.03
Invert	0.0916	0.1086	1.06
Ethyl alcohol	0.0667	0.0806	0.76
Sodium acetate	0.0663	0.0805	0.58

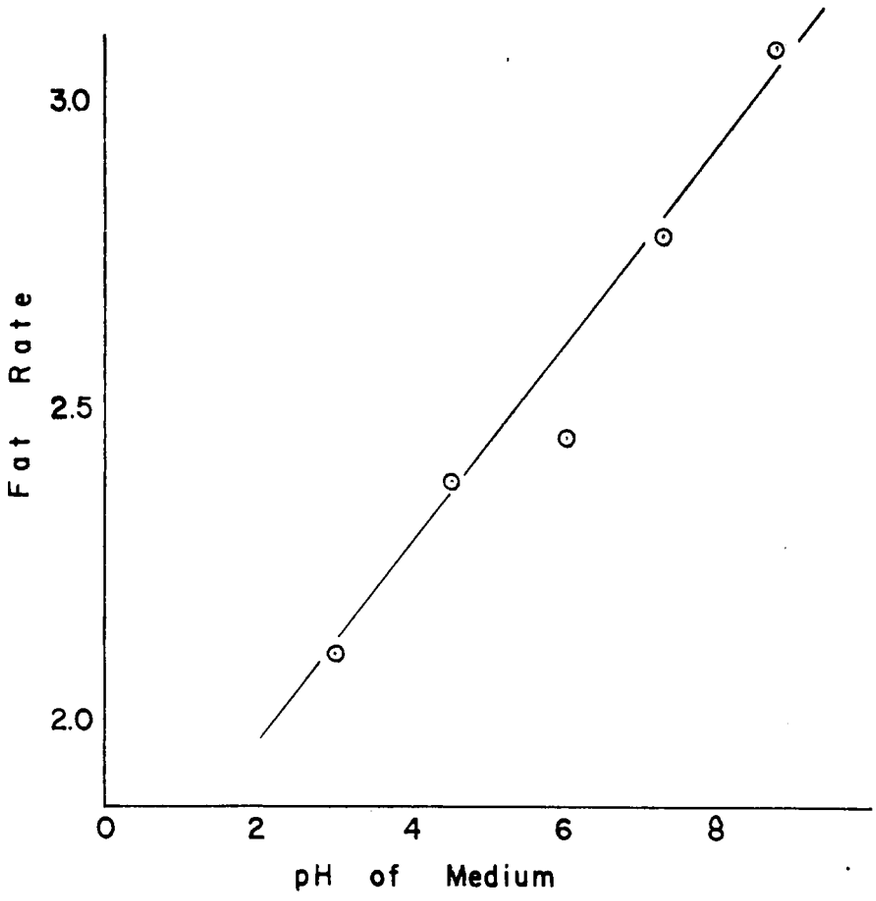


Figure 4. Variation of fat rate with pH of fattening medium

grown and fattened in fructose than in glucose.

Sugar Degradation Products Recent investigations (*1, 7, 12, 13*) have demonstrated that fatty acids are synthesized from a two-carbon compound such as ethyl alcohol or acetate. Sneed (*11*) has demonstrated that glycerol stimulates fattening in *Aerobacter cloacae*. Several experiments were performed in an effort to determine whether or not these intermediates would accelerate fat synthesis.

Ethyl alcohol and sodium acetate, in concentration equimolar as to carbon content with the sugars, were included with the experiment on sugars and the results are included in Table V. Although in both instances an appreciable amount of fat was formed, the fat contents were much lower than when sugar was the substrate. The pH of the sodium acetate samples rose from an initial pH of 7.55 to 9.12 at 38 hours presumably owing to acetate utilization. The remaining samples showed a decrease in pH to about 5.5.

In a second experiment, glycerol and sodium acetate were used as adjuncts to corn sugar, replacing 10% of the normal sugar concentration on a carbon basis. The samples containing glycerol showed dry weights and fat contents (41.4 %) after 29 hours almost identical to the corn sugar control. This led to the conclusion that glycerol in the medium was of no added value in fat synthesis. The yeast suspensions containing acetate were adjusted initially to pH 4.78. The result was complete inhibition of the cell growth. The dry weight of yeast did not increase between 8.5 and 29 hours.

White and Werkman (*14*) similarly found that the amount of fat formed by *S. cerevisiae* from acetate fell off sharply as the initial pH was decreased from pH 6.5 to 5.5.

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Figure 5. Effect of high sugar concentrations on fat formation