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Isaac Isaac Peters
Iowa State University

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THE LIPOLYTIC ENZYME SYSTEM OF MYCOTORULA LIPOLYTICA

by

Isaac I. Peters

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Signature was redacted for privacy.

In charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

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INTRODUCTION

The present trend in the dairy industry is to safeguard the quality of its products by pasteurization. In addition to destroying all infectious disease-producing bacteria and a large proportion of the other microorganisms present, this treatment inactivates some of the enzymes naturally present in milk, one of them being the fat-splitting enzyme lipase. Milk lipase is essential in the procedure commonly used for the manufacture of blue cheese. This enzyme hydrolyzes the butterfat to give free fatty acids, the lower ones of which contribute flavor directly and also serve as the substrate for ketone formation by Penicillium roqueforti.

A study was undertaken to select a suitable microorganism possessing pronounced lipolytic properties, the enzyme of which could be prepared and added to pasteurized milk in place of the inactivated milk lipase. The organisms in question also must be non-pathogenic, essentially colorless, preferably non-fastidious in growth requirements and preferably producing most of the lipolytic enzyme so it is available exocellularly. The lipase produced should exhibit its optimum activity in the range of pH 4.5 - 6.5 in which blue cheese ripening takes place.

After preliminary studies on a number of lipolytic microorganisms, the yeast Mycotorula lipolytica, Harrison, also known as Torula lipolytica, Jacobsen (Harrison, 1928) and as Candida lipolytica (Diddens and Lodder, 1940; Chinn, 1946), was selected for detailed study as its lipolytic enzyme system seemed to have the desired characteristics for use in the manufacture of blue cheese from pasteurized milk.

STATEMENT OF PROBLEM

The work herein reported was undertaken to obtain information concerning the lipolytic enzyme system of Mycotorula lipolytica and the suitability of this enzyme system for use in the manufacture of blue cheese. The study was divided into the following five sections:

The selection of M. lipolytica for production of lipase.

Factors influencing the production of lipase by M. lipolytica.

Factors influencing the activity of M. lipolytica lipase.

Concentrating of M. lipolytica lipase.

The influence of the addition of various amounts of M. lipolytica lipase upon the ripening of blue cheese made from pasteurized homogenized milk.

REVIEW OF LITERATURE

Lipolysis by Achromobacter lipolyticum, Alcaligenes lipolyticus, Pseudomonas fragi, and Mycotorula lipolytica

Among the many lipolytic microorganisms reported in the literature only four of those known to be highly lipolytic were considered in this study and the review of the lipolytic microorganisms is confined largely to a consideration of these four species.

Achromobacter lipolyticum was identified by Huss (1908) as causing rancidity in milk. He found it to have an optimum growth temperature of 35°C. Collins (1933) observed that cotton-seed oil globules in nile-blue sulfate agar plates were rapidly and completely hydrolized beneath the colonies, showing that a diffusible lipolytic enzyme was produced by the cells. The simple triglycerides from tripropionin to trimyristin, inclusive, were hydrolyzed. Fouts (1939) found Achr. lipolyticum grew well in cream at acidities up to 1.0 percent and caused hydrolysis of butterfat. However, the acid number of the fat decreased with increasing acidities of the cream. Long and Hammer (1938, 1939) observed the appearance of rancidity and an increase in free fatty acids in butter samples inoculated

with this organism and held at either 10 or 21°C. over a total period of up to 30 days.

Long (1936) proposed the name Alcaligenes lipolyticus for a lipolytic aerobic bacterium, previously known as Bacillus abortus var. lipolyticus and as Bacterium lipolyticus (Evans, 1917, 1918). He studied 21 cultures, all of which hydrolyzed the cottonseed globules below the colonies in nile-blue sulfate agar, and 20 of which hydrolyzed the oil globules for a considerable distance beyond the edge of the colonies. All of the cultures examined by him hydrolyzed triisovalerin, tricaproin tricaprylin, tricaprin, trilaurin and triolein. Tripropionin and tributyrin were hydrolyzed by most of the cultures. Trivalerin, triheptylin, trimyristin, tripalmitin and tristearin were not hydrolyzed. The optimum growth temperature of Alc. lipolyticus was below 40°C. Long and Hammer (1938, 1939) found butter inoculated with Alc. lipolyticus cultures developed rancidity in from 10 to 18 days when stored at 21°C., and acid degree values rose with holding time of butter.

Pseudomonas fragi was named and characterized by Hussong (1932). He characterized the organism as psychrophilic, aroma-producing and capable of hydrolyzing fat. Variations in fat-splitting abilities were encountered with different cultures of this organism. The bacterium

was isolated from rancid butter and various other dairy products. Long (1936) found Ps. fragi to produce a diffusible lipolytic enzyme with fat hydrolysis extending over a considerable area around the growth zone. Tripropionin and tributyrin were hydrolyzed by all of the cultures studied except by a few that failed to grow on the medium. Trivalerin and triolein were hydrolyzed by a majority of the 42 cultures studied, while triisovalerin, tricaproin, tricaprylin and tricaprin were hydrolyzed by some cultures but not by others. Triheptylin, trilaurin, trimyristin, tripalmitin and tristearin were not attacked. Long and Hammer (1938, 1939) inoculated butter with Ps. fragi cultures and observed development of a rancid odor after holding from 2 to 6 days at 21°C. They also found that the free fatty acids increased in the inoculated butter samples held at 10°C. with increasing holding periods, indicating hydrolysis of butterfat.

Harrison (1928), in his classification of torulae, characterized Mycotorula lipolytica as capable of hydrolyzing butterfat. Long (1936) made the same observation on a number of cultures isolated from raw milk and cheesy, rancid butter. Eight out of eleven cultures studied by him hydrolyzed all of the fat globules in the agar below the growth, while the remaining three hydrolyzed only a part of them. Seven cultures hydrolyzed the fat globules

beyond the colony growth zone, while four failed to do so. All of the cultures hydrolyzed tripropionih, tributyrin, trivalerin, triisovalerin, tricaprylin, tricaprin and triolein; tricaproin and trilaurin were hydrolyzed only slightly. Triheptylin was attacked by some cultures but not by others, while trimyristin, tripalmitin and tristearin were not hydrolyzed. Fouts (1939) found that M. lipolytica grew well in cream with titratable acidities up to 1.0 percent, and caused lipolysis in cream with acidities up to 2.08 percent. This organism showed increased growth in the presence of butter cultures and exceeded Achr. lipolyticum and Alc. lipolyticus in lipolytic activity at cream acidities close to 1.0 percent. Long and Hammer (1938, 1939) inoculated butter samples with a pure culture of M. lipolytica and observed detectible rancidity in from 1.5 to 3.5 days at 21°C. The acid degree values rose with prolonged holding periods up to 30 days at a temperature of 10°C. This organism was more lipolytic in well-worked butter than was Ps. fragi, and was equal to or slightly stronger than Achr. lipolyticum in this respect.

Chinn (1946) isolated Candida lipolytica from cream and butter and found it produced the same defects in dairy products as were reported earlier by Long (1936). The organism was aerobic and its optimum growth temperature

was between 21 and 30°C.

The Influence of Growth Conditions Upon Lipase Production by Microorganisms

The only organism for which published information could be found concerning the cultural conditions which influence lipase production was P. roqueforti. Weissbrodt (1927) was able to increase lipase production of P. roqueforti by substituting ammonium chloride for sodium nitrate in Czapek's medium as modified by Dox. Naylor, Smith and Collins (1930) were able to verify the above results. Thibodeau (1940) observed that sugar retarded production of the lipase of P. roqueforti, while the presence of both agar and organic nitrogenous compounds increased the production of lipase. According to Weissbrodt (1927) and Naylor, Smith and Collins (1930), lipase production of P. roqueforti was highest at pH 4.5 in Czapek's modified medium.

Factors Influencing the Action of Microbial Lipases

Collins and Hammer (1934) examined a large number of lipolytic microorganisms by the nile-blue sulfate method and found that, in general, each of the organisms used had much the same action on various natural and hydrogenated fats. They also observed that the hydrolysis of simple

triglycerides of saturated fatty acids was more difficult as the molecular weight increased. However, triolein was comparatively easily hydrolyzed. According to Balls, Matlack and Tucker (1938) triolein is hydrolyzed as readily as is a nine carbon saturated fatty acid, although it contains 18 carbons.

Avery and Cullen (1920) found pneumococcus lipase exhibited optimum activity at pH 7.8. The same was true for the lipase of hemolytic streptococci, according to Stevens and West (1922). Gorbach and Guentner (1932) found the lipase of beer-yeast to be most active at pH 6.6 - 6.8. Thibodeau (1940) reported lipase from P. roqueforti showed optimum activity at pH 5.3 - 7.5.

Gorbach and Guentner (1932) found beer-yeast lipase showed optimum activity at 30°C. Higher temperatures were detrimental to the enzyme. The same workers observed also that the rate of lipase activity decreased with time. They also showed that the amount of fat hydrolyzed was not directly proportional to the amount of beer-yeast lipase present, but that there was a point of optimum activity per unit of lipase.

Methods for Concentration and Purification of Lipase

Ostwald and Siehr (1937) pointed out the suitability of foam-adsorption method for separation and concentration

of colloidal molecular substances; they believed their method superior to other methods applicable to enzyme systems. Ostwald and Mischke (1940) were able to concentrate pancreatic lipase solutions at pH 4.5, using nitrogen gas as the bubbling agent. According to Babel (1947), this method was applied with some success in concentrating microbial lipases.

Gyotoku and Terashima (1930) were able to precipitate lipases of blood, pancreas, and stomach by the use of 55 - 60 percent concentration of ammonium sulfate, while Glick and King (1933) used, with varying success, magnesium sulfate, half and fully saturated ammonium sulfate and 10 percent sodium chloride.

Procedures for Manufacture of Blue Cheese

Methods for the manufacture of blue cheese from raw cows' milk have been described by Thom, Matheson and Currie (1914), Matheson (1921) and by Goss, Nielsen and Mortensen (1935). Lane and Hammer (1936) showed that homogenization of raw milk resulted in faster ripening and in more luxurious mold growth than if nonhomogenized milk was used. Later (1938) the same workers reported that blue cheese made from pasteurized homogenized milk gave better results than raw nonhomogenized milk, but not as good as raw homogenized milk. They concluded that milk lipase definitely aided in

cheese ripening. Fabricius and Nielsen (1941) were able to produce a satisfactory blue cheese by using a combination of heat-vacuum treatment of milk, namely, 165 - 175°F. and 19 inches of vacuum, their procedure destroying most of the undesirable microorganisms in the milk but not inactivating the milk lipase. Irvine (1938) added a commercial lipase preparation, namely steapsin (Ontario, 1937), at the rate of 0.5 to 1.0 gm. per 100 pounds of raw milk. He observed acceleration of fat hydrolysis and quicker ripening, but a bitter flavor in the cheese resulted. Similar results were obtained by Coulter and Combs (1939), who used the same procedure as Irvine.

EXPERIMENTAL

Methods

Source of cultures

All cultures used were from the stock culture collection of the Department of Dairy Industry, Iowa State College.

Medium for carrying cultures

Stock cultures were carried in tubed litmus milk and on tryptone-glucose-extract agar slants.

Medium for lipase production

The composition of the nutrient broth used for lipase production was as follows:

Bacto peptone	5 gm.
Beef extract	3 gm.
Glucose	0.5 gm.
Distilled water	500 ml.
0.6 m $Na_2HPO_4.12\ H_2O$	230 ml.
0.3 m $C_6H_8O_7.H_2O$	270 ml.
Reaction	pH 4.5

Inoculation of medium and growth of cultures

One drop of 24-48 hr. culture in litmus milk was added to 200 ml. of sterile nutrient broth in all trials in which cultures were examined at given growth intervals, while one ml. quantities of growing cultures were used in

the remaining trials. Cultures were grown at 30°C. unless stated otherwise. In the earlier experiments the cultures for lipase production were shaken twice daily, while in later trials the shaking was omitted, except at the time of sampling.

Determination of cell count

Tryptone-glucose-extract agar was used for pouring plates (American Public Health Association, 1941). All plates were counted after holding for 48 hrs. at 30°C.

Staining of cells

Methylene blue or gentian violet stain was used in preparing slides for microscopic examination of cells.

Determination of pH

These were made either with a Leeds and Northrup quinhydrone electrode and saturated calomel half cell or with a Leeds and Northrup universal glass-electrode potentiometer.

Determination of lipolytic properties of microorganisms

For qualitative studies of lipolytic properties of cultures a modification of the procedure used by Jensen and Grettie (1937) was followed. Butterfat emulsion was

added in 0.5 ml. quantities (1.2 gm. butter oil, 0.25 gm. agar, 50 ml. water) to each plate before pouring with nutrient agar. Nile-blue sulfate solution (1:500) was added to the melted agar at the rate of 5 ml. per 100 ml. of agar. Cultures were streaked on the solidified surface of the agar and the plates were held at 21°C. for four days. The presence of blue fat globules below and around the colonies was taken as indicative of fat hydrolysis.

Determination of oxidative properties of microorganisms

For studies of oxidative properties of cultures a modification of the procedure of Jensen and Grettie (1937) was used. Except for the omission of nile-blue sulfate the substrate was the same as for determination of lipolysis. After incubation the plates were flooded with a 0.5 percent aqueous solution of dimethylparaphenylenediamine hydrochloride. The presence of rose-red colonies was considered as positive test for oxidative action by the organism in question.

Quantitative determination of lipase activity

A butterfat-agar emulsion was prepared having the following composition:

Butterfat	5.0 gm.
Agar	0.25 gm.
Distilled water	80 ml.
0.6^{m} $Na_2HPO_4.12\ H_2O$	10.5 ml.

0.3m $C_6H_8O_7.H_2O$	4.5 ml.
Formaldehyde	1:1500
Reaction	pH 4.5

The water, buffer and agar were mixed, heated to boiling, cooled until the agar had solidified, and tempered over night to 45°C. Butterfat was melted, heated to boiling and also tempered to 45°C. over night. The butterfat and formaldehyde were added to the agar, the mixture was shaken well and homogenized twice with a hand homogenizer so as to obtain a stable emulsion. The emulsion was transferred in 50 ml. quantities into clean 6 oz. screw cap bottles, this being the quantity which was used for each determination of lipase activity. Inoculations for lipase determination were made at the rate of two percent (1 ml.) of culture or supernatant, as the case may be, followed by a 48 hr. incubation period at 37°C. with twice-a-day shaking. A control was run on every active culture. This control was identical in all respects to the active sample, except that the culture or supernatant had been heated to boiling for 15 minutes in order to destroy the lipase enzyme.

Titrations were made in duplicate on 10 gm. portions of the emulsions to which the heated and unheated material had been added. To each weighed sample was added 50 ml. of a 50:50 mixture of neutral 95 percent ethyl alcohol and diethyl ether. The free fatty acids were titrated with N/20 potassium hydroxide in absolute methyl alcohol, accord-

ing to the method of Bird (1946), using phenolphthalein as indicator. The difference in titration values between the active sample and the control was regarded as due to lipase activity. Lipase activity was calculated and expressed in acid degrees, which are defined as the number of milliliters of N/1 sodium hydroxide required to neutralize the free fatty acids in 100 gm. of fat (Association of Official Agricultural Chemists, 1940).

Preparation of lyophilized material

Three-day old nutrient broth cultures of M. lipolytica in 200 ml. quantitites were placed in a 500 ml. round-bottom flask with ground glass stopper. The culture was frozen while rotating the flask in an acetone-dry ice bath. The flask containing the frozen culture was evacuated by use of a vacuum pump. The evaculation of air resulted in sublimation of the ice in the frozen culture. The water vapors were extracted by continuous suction and condensed and frozen in another flask which was held in an acetone-dry ice bath.

Concentration of enzyme by foaming

Three-day old nutrient broth cultures of M. lipolytica to be used for studies on adsorption by foaming were centrifuged and the supernatant placed in 200 ml. or 100 ml.

quantities into 500 ml. or 225 ml. gas washing bottles, respectively. Foaming was produced by bubbling carbon dioxide (compressed in a cylinder) through the supernatant at from 50 to 75 mm. mercury pressure. The foam was collected in graduated cylinders of convenient size to permit the collection of liquid foam desired. In the experiments on repeated foaming the third and fourth fractionated liquid foam portions were diluted by adding distilled water, acidified with hydrochloric acid to pH 4.0, to obtain the desired volume, namely 200 ml., for fractionation.

Precipitation of lipase with ammonium sulfate and ethyl alcohol

Three-day old cultures were centrifuged and the supernatant used in 400 ml. quantities. To 400 ml. of supernatant in a 1 liter separatory funnel was added ammonium sulfate to the saturation point (about 200 gm.). Next 130 ml. of 95 percent ethyl alcohol was added and the mixture well shaken. The flocculent precipitate formed gathered at the water-alcohol interface. After first drawing off the aqueous layer, the precipitate, together with the alcohol layer, was decanted onto a previously weighted hardened filter paper, supported in a Buchner funnel. Suction was applied to the flask and the alcohol filtered off. Ten ml. of diethyl ether were added to remove the remaining alcohol.

The filter paper was freed from the last traces of ether by placing it at 37°C. for a few hours. The dry precipitate on the filter paper was weighed and used for lipase activity measurement.

Preparation of cell-free filtrate for use in blue cheese

Three-day old nutrient broth cultures of M. lipolytica were centrifuged and filtered through an ultra-fine glass filter. This procedure resulted in a cell-free preparation and was used as a source of lipase in making cheese. Lipase activity measurements per ml. of filtrate were made prior to its use.

Cheese making procedure

Regular pasteurized, homogenized milk was used in all experiments in quantities of 105-110 lbs. per vat. Three or four vats were used at one time and conditions kept as uniform as possible throughout the process. The vat contents were kept at 90°F. from the time of adding the culture until the curd was hooped. One percent starter was used and both rennet (90 ml./1000 lbs.) and lipase were added 30 minutes later. The curd was cut 70 minutes after setting and held for 2 hours, with some stirring every 30 minutes. One percent salt and 0.01 percent mold powder were added to the curd at time of hooping. Dry salting

at the rate of 5 lbs. total per 100 lbs. of curd was done daily for 4 days, after which time the cheese was scewerd and placed in the ripening room at 10°C. and a relative humidity of approximately 90 percent. The cheese was held for 12 weeks.

Scoring of cheese

The cheese were examined and scored after 4 weeks and 12 weeks for visual mold growth, positive flavor, and defects. A score of 10 was considered perfect in each of the three items under consideration.

Analysis of cheese for total volatile acidity

The total volatile acidity of the cheese was determined by the method of Lane and Hammer (1938).

Analysis of cheese for moisture

The moisture content of the cheese was determined on 10 gm. samples in a Brabender Semi-Automatic Moisture Tester at 142°C. Weighings were continued until the weights at 10 minute intervals became constant.

Analysis of cheese for fat

The fat content of the cheese was determined by a modification of the procedure of the Association of Official

Agricultural Chemists (1940), which modification made it suitable for use with the Mojonnier tester. The modification included the use of 5 ml. of water instead of 9 ml., the transfer to a Mojonnier fat extraction flask instead of a Röhrig tube, and the use of 10 ml. of ethyl alcohol with the 25 ml. of ethyl ether and 25 ml. of petroleum ether. The ether-fat mixture was poured into a weighed Mojonnier fat dish after being centrifuged 30 turns in 30 seconds. The second extraction consisted of 25 ml. of ethyl ether and 25 ml. of petroleum ether, and was poured into the same dish. After the ether was evaporated, the dish and fat were dried 5 minutes at 100°C. under 20 inches of vacuum, cooled in the Mojonnier dessicator 7 minutes and weighed.

Chloride content of the cheese

The total chloride content of the cheese was determined by the method of the Association of Official Agricultural Chemists (1940) with one modification, namely the use of 50 ml. of N/10 silver nitrate instead of 25 ml.

Results

The selection of M. lipolytica for production of lipase

A comparison of their relative activity upon butterfat

was made between pure cultures of Achr. lipolyticum, Alc. lipolyticus, Ps. fragi and M. lipolytica. Nile-blue sulfate was used as indicator for hydrolysis of fat and para-aminodimethylaniline monohydrochloride as indicator for oxidation of fat.

M. lipolytica was found to possess the most pronounced lipolytic properties, followed by Achr. lipolyticum, Ps. fragi and Alc. lipolyticus, respectively. All cultures except Alc. lipolyticus showed hydrolysis of fat globules beyond the growth zone of the colonies. The oxidative action of the organisms was highest in the case of Achr. lipolyticum, followed by Alc. lipolyticus, M. lipolytica and Ps. fragi.

To obtain more quantitative information on their lipolytic properties Ps. fragi, Achr. lipolyticum, and M. lipolytica were inoculated into 1.5 l. quantities of unbuffered nutrient broth at pH 6.6, and grown for 48 hrs. The cells were removed by centrifuging and ground with mortar and pestle for 15 minutes using finely ground pyrex glass in amounts of double the weight of the moist cells. The pasty mixture was diluted with four times its weight of distilled water and centrifuged at 1500 r.p.m. for 15 minutes. The supernatant portion was used to determine endocellular lipase activity, while the broth from which the cells had been centrifuged was used to determine exocellular lipase activity. The results of this trial are shown in Table 1.

Table 1. . The Endo and Exocellular Lipase Activity of Achr. lipolyticum, Ps. fragi and M. lipolytica

Culture	Acid degrees in Supernatant (total)*	Acid degrees in wet cells (total)*	Weight of wet cells (total)
Achr. lipolyticum	150	4	1.8 gm.
Ps. fragi	1,800	15	3.1 gm.
M. lipolytica	4,000	125	3.0 gm.

* Calculated from increments used.

The results showed that M. lipolytica exhibited more lipase activity than either of the other two cultures, both in the supernatant portion and in the wet cells. Consequently this culture was selected for further study as a possible source of lipase to be used in blue cheese. The high total lipase activity of the supernatant broth in comparison to the relatively low-activity of the cells led to the study of the supernatant as the source of the enzyme system. Later results of ten unselected 3-day old cultures, grown in buffered nutrient broth with initial pH 4.5, showed that the supernatant retained about 70 percent of the total lipase activity of the uncentrifuged culture.

Factors influencing the production of lipase by M. lipolytica

Composition of medium. In a preliminary determination of the medium characteristics necessary for optimum lipase production a comparison was made of five media with the following compositions:

Medium 1. Unbuffered nutrient broth at pH 6.6.

Medium 2.		
	$(NH_4)_2 HPO_4$	0.5 gm.
	Glucose	0.05 gm.
	Yeast extract	0.01 gm.
	Thiamin	0.005 gm.
	Salt mixture A*	1 ml.
	Salt mixture B**	1 ml.
	Distilled water to make	100 ml.
	Reaction adjusted to	pH 7.2.

*Salt mixture A

K_2HPO_4	25 gm.
KH_2PO_4	25 gm.
Distilled water to make 250 ml.	

** Salt mixture B

$MgSO_4.7H_2O$	10 gm.
NaCl	0.5 gm.
$FeSO_4.7H_2O$	0.5 gm.
$MnSO_4.4H_2O$	0.5 gm.
Distilled water to make 250 ml.	

Medium 3.

$Na_2HPO_4.12\ H_2O$	0.5 gm.
Glucose	0.05 gm.
Tryptophane	0.04 gm.
Cysteine	0.04 gm.
Leucine	0.04 gm.
Lysine	0.04 gm.
Glutamic acid	0.02 gm.
Yeast extract	0.01 gm.
Thiamin	0.005 gm.
Salt mixture A*	1 ml.
Salt mixture B**	1 ml.
Distilled water to make	100 ml.
Reaction adjusted to pH 7.2	

Medium 4. Regular pasteurized homogenized milk

Medium 5.

Skim milk powder	10 gm.
Distilled water to make	100 ml.

Inoculations were made into 200 ml. quantities of sterile medium and the incubation procedure conducted as outlined under "Methods" with twice-a-day shaking of cultures. The results obtained on 2-day old cultures are shown in Table 2. The outstanding superiority of the nutrient broth is apparent. The failure of the other media to support the production of lipase opens some interesting possibilities for study of factors in defined media which favor lipase production.

The trial was repeated twice with media 1 and 2 and a nutrient broth ten times the strength of the original, desig-

Table 2. The Influence of Different Growth Media Upon Lipase Production and Growth of M. lipolytica (48-hour cultures)

Type of growth medium	Acid degrees per ml. of supernatant	Count per ml. (in millions)
1. Nutrient broth	8	7.1
2. Synthetic with $(NH_4)_2HPO_4$	0	2.2
3. Synthetic with $Na_2HPO_4.12H_2O$	0	0.3
4. Past. homogenized milk	0	0.5
5. Reconstituted skim milk	0	40.0

Table 3. The Influence of Different Growth Media Upon the Lipase Production and Growth of M. lipolytica

Type of growth medium	Acid degrees per ml. of culture		Count per ml. (in millions)	
	Trial 1	Trial 2	Trial 1	Trial 2
48-hour cultures				
1. Nutrient broth	11.0	13.0	12.0	17.0
2. Synthetic with $(NH_4)_2HPO_4$	0.0	0.0	5.0	4.5
3. Nutrient broth 10X	16.5	22.0	13.0	25.0
96-hour cultures				
1. Nutrient broth	6.5	9.0	12.0	8.7
2. Synthetic with $(NH_4)_2HPO_4$	2.0	7.0	15.0	12.9
3. Nutrient broth 10X	23.0	24.0	6.5	20.0
144-hour cultures				
1. Nutrient broth	2.0	2.0	13.0	16.0
2. Synthetic with $(NH_4)_2HPO_4$	0.5	4.0	11.0	10.8
3. Nutrient broth 10X	8.0	27.0	23.0	18.0

nated as 10X. Tests were run on 48, 96, and 144-hour cultures using the same method as in the preceding study. Table 3 shows the results of these trials.

The results in Table 3 show that nutrient broth was the best growth medium of those tested for the stimulation of lipase production. Increasing the strength of the nutrient broth resulted in increased lipase production, but not at a rate proportional to the increase in concentration. No correlation between the amount of lipase produced and the number of cells present was found. The amount of lipase did not continue to increase with the age of the culture, but rather declined, in most instances, after 96 hours growth time. The regular nutrient broth was selected as growth medium for further studies of lipase production by M. lipolytica, despite the higher lipase activity resulting from use of the nutrient broth 10X, because of the greater economy and ease of handling of the former.

Two trials were run using buffered nutrient broth at pH 4.5 with 0.0, 0.01, 0.05, 0.25, and 1.0 percent of glucose added to different lots. These trials were made after preliminary results had shown the value of lower acid reaction than pH 6.6 and 7.2 as used before. Examinations were made for lipase production, cell count, and morphological characteristics of cells after growth for 48, 72, 96, and 144 hours. Results are shown in Table 4.

Table 4. The Influence of Glucose Concentration upon Lipase Production, Cell Count, and Shape of Cells

Percent glucose	Acid degrees per ml. culture	pH of medium	Count per ml. (in millions)	Predominant shape of cells
Trial 1				
48-hour cultures				
0.00	52	4.6	8.3	Long, slender
0.01	58	4.6	9.4	Long, slender
0.05	51	4.6	9.4	Long, slender
0.25	40	4.6	14.0	Long, oval and slender
1.00	28	4.6	15.0	Short, oval
72-hour cultures				
0.00	52	4.8	No	Long, oval and slender
0.01	42	4.8		Short and long oval
0.05	45	4.8	counts	Short and long oval
0.25	38	4.7		Short and long oval
1.00	18	4.5	obtained	Short and long oval
96-hour cultures				
0.00	34	4.9	No	
0.01	37	4.9		No results
0.05	49	4.9	counts	
0.25	21	4.7		
1.00	21	4.5	obtained	
Trial 2				
48-hour cultures				
0.00	16	4.7	5.1	Long, slender
0.01	14	4.7	4.8	Long, slender
0.05	14	4.7	5.4	Long, slender
0.25	14	4.7	8.7	Long, slender, short oval
1.00	5	4.7	7.9	Short oval
72-hour cultures				
0.00	30	4.8	3.5	Long, slender
0.01	23	4.8	3.3	Long, slender
0.05	23	4.8	3.5	Long, slender
0.25	23	4.7	8.7	Short, oval
1.00	12	4.6	8.0	Short, oval
96-hour cultures				
0.00	24	4.9	4.2	Long, slender
0.01	24	4.9	4.7	Long and short oval
0.05	24	4.9	5.1	Long and short oval
0.25	19	4.7	7.2	Long and short oval
1.00	16	4.6	6.6	Long, oval
144-hour cultures				
0.00	22	5.2	5.2	Long, oval
0.01	21	5.2	6.5	Long and short oval
0.05	22	5.2	6.9	Long and short oval
0.25	19	5.0	8.5	Long and short oval
1.00	17	4.8	7.4	Long oval

The influence of glucose upon the production of lipase by M. lipolytica was similar in the two trials conducted. Absence of glucose or the presence in low concentrations resulted in earlier and more rapid lipase production. Concentrations of 0.25 and 1.00 percent glucose retarded the production of lipase, the effect being more pronounced at the higher concentration of the suger. The amount of glucose present influenced also the growth rate of the cells. Higher counts were obtained with increasing concentration of sugar. The shape of the cells also was affected, the slower rate of growth resulting in a predominance of long oval and long slender cells, as compared with short oval cells when growth was more rapid. The presence of glucose up to 0.05 percent was more satisfactory than greater concentrations for highest lipase production over the period studied.

pH of medium and type and concentration of buffer. To determine the effect of pH upon lipase production, unbuffered nutrient broth in 200 ml. quantities was adjusted to pH levels ranging from pH 3.5 to 8.3, using N/1 sodium hydroxide or N/1 hydrochloric acid as reagents. A second series was prepared with 0.05 m disodium phosphate plus monosodium phosphate added. Cultures were grown at 30°C. in the regular manner, shaken twice daily, and examined for lipase production and cell counts after 40, 64, 88, and 136 hours. Results are shown in Table 5.

Table 5. The Influence of pH and Buffering Upon Lipase Production and Growth of *M. lipolytica*

pH of medium		Acid degrees per ml. of culture at				Colony count per ml. (in millions) at			
Initial	Final*	40 hrs.	64 hrs.	88 hrs.	136 hrs.	40 hrs.	64 hrs.	88 hrs.	136 hrs.
Unbuffered									
3.5	4.0	0	3.0	2.0	7.5	0.4	0.6	0.4	1.6
4.5	7.3	0	10.5	6.0	6.0	0.6	20.0	15.0	41.0
5.5	6.3	0	8.8	8.0	9.0	1.5	19.0	23.0	3.7
6.5	6.7	0	9.2	10.0	5.0	2.0	23.0	34.0	28.0
7.5	7.8	3.0	8.0	5.5	8.0	2.2	19.0	30.0	52.0
8.3	8.3	0	5.0	11.0	2.0	1.2	18.0	32.0	12.0
Buffered									
3.5	4.2	0	0	5.0	15.0	0.1	0.6	1.0	2.6
4.5	6.0	0	4.0	12.5	18.0	0.8	7.0	8.1	5.5
5.5	7.5	0	9.5	19.0	3.0	1.1	5.3	9.5	40.0
6.5	7.8	3.0	7.5	2.5	4.8	2.2	20.0	23.0	24.0
7.5	7.9	4.8	11.0	5.0	0	3.0	16.0	40.0	32.0
8.3	8.3	0	0	6.0	0	1.2	9.6	23.0	34.0

* At 136 hours.

The results of this trial show that the pH level of the medium in both the unbuffered and buffered series plays an important part in lipase production. Highest lipase production was obtained in the unbuffered medium when the initial pH was 4.5 - 6.5 and in the buffered medium when the initial pH was either 4.5 or 5.5. A shifting of the pH towards the alkaline side as a result of growth was observed in all media except those initially at pH 8.3. Initial growth of cells was most rapid at pH 6.5 and 7.5. Again lipase activity did not parallel cell population.

In the studies designated to determine the suitability of different buffers in the medium in which the organisms were grown to produce lipase, unbuffered nutrient broth of double strength was prepared and used in 100 ml. quantities. Four 100 ml. quantities were adjusted to pH 7.5, using N/1 sodium hydroxide, while six other lots were adjusted to pH 5.5, using N/1 hydrochloric acid and equal volumes of buffer at pH 7.5 or 5.5 were added. Disodium phosphate and monopotassium phosphate were added in amounts to give concentrations of 0.225 m and 0.075 m, both at pH 7.5 and pH 5.5. Disodium phosphate and citric acid were added in the same manner, while sodium acetate and acetic acid was used only at pH 5.5 in concentrations of 0.225 m. and 0.075 m. After inoculation the sterilized lots were incubated at 30°C. and shaken twice daily. Examinations for lipase

activity and cell count were made at 48, 72, 96, and 144 hours. Results of two trials are shown in Table 6.

The results of the two trials show that the cultures grown in the presence of sodium acetate and acetic acid produced the least lipase, especially at the buffer concentration of 0.225 m. The other two buffer pairs compared favorably, both being more suitable for lipase production at pH 5.5 than at pH 7.5. The higher level of concentration of these two buffer pairs proved more desirable since less shifting of pH towards the alkaline side occurred over the 6 day growth period. Cultures grown at the lower buffer concentration showed more rapid growth than at the higher concentration, but shifted in reaction more rapidly. Based on the results of these two trials disodium phosphate and citric acid buffer at a concentration of 0.225 m. was selected for further studies.

In further studies on the effect of pH upon lipase production made after the useful influence of the phosphate-citrate buffer upon lipase production had been demonstrated, a series of 200 ml. quantities of nutrient broth media were adjusted to pH levels of 3.5 to 8.5 by means of disodium phosphate and citric acid with final buffer concentration of 0.225 m. Inoculations were made and the cultures grown at 30°C. without twice-a-day shaking. Examinations for lipase production, cell count, and shape of cells were made

Table 6. The Influence of Various Buffer Pairs, at Different Concentratio
Production and Growth of M. lipolytica

Buffer Pairs	Buffer concentration	pH of medium: Initial	pH of medium: Final*	Acid degrees per ml. of culture after growth periods of: 48 hrs.	72 hrs.	96 hrs.	144 hrs.
				Trial 1			
$Na_2HPO_4.12H_2O$ + KH_2PO_4	0.225	7.5	7.6	11.0	12.5	8.0	8.5
	0.075	7.6	7.6	16.0	14.0	6.0	5.8
$Na_2HPO_4.12H_2O$ + $C_6H_8O_7.H_2O$	0.225	7.6	7.6	12.0	10.0	6.8	3.0
	0.075	7.6	7.7	4.5	8.5	4.5	3.5
$Na_2HPO_4.12H_2O$ + KH_2PO_4	0.225	5.5	5.7	16.0	13.5	13.5	15.5
	0.075	5.8	6.2	6.0	13.0	20.0	11.5
$Na_2HPO_4.12H_2O$ + $C_6H_8O_7.H_2O$	0.225	5.7	6.0	17.0	23.0	22.0	15.0
	0.075	6.1	6.7	17.0	22.0	22.8	15.0
$C_2H_3OONa.3H_2O$ + C_2H_3OOH	0.225	5.6	6.6	0.0	1.0	1.0	10.0
	0.075	5.8	7.8	11.8	13.5	17.0	1.5
				Trial 2			
$Na_2HPO_4.12H_2O$ + KH_2PO_4	0.225	7.5	7.7	32.0	30.0	13.0	15.0
	0.075	7.5	7.9	31.0	23.0	16.0	11.0
$Na_2HPO_4.12H_2O$ + $C_6H_8O_7H_2O$	0.225	7.5	8.0	21.0	26.0	50.0	22.0
	0.075	7.5	8.0	31.0	20.0	24.0	8.0
$Na_2HPO_4.12H_2O$ + KH_2PO_4	0.225	5.5	6.0	50.0	50.0	44.0	47.0
	0.075	5.5	6.4	42.0	45.0	32.0	34.0
$Na_2HPO_4.12H_2O$ + $C_6H_8O_7H_2O$	0.225	5.5	6.2	40.0	45.0	40.0	36.0
	0.075	5.5	6.4	52.0	44.0	42.0	35.0
$C_2H_3OONa.3H_2O$ + C_2H_3OOH	0.225	5.5	8.4	3.0	14.0	12.0	4.0
	0.075	5.5	8.8	31.0	32.0	23.0	19.0

* At 144 hours.

Buffer Pairs, at Different Concentrations and pH levels Upon Lipase Production and Growth of M. lipolytica

um :	Acid degrees per ml. of culture after growth periods of:				Count per ml. (in millions) after growth periods of:			
al*:	48 hrs.	72 hrs.	96 hrs.	144 hrs.	48 hrs.	72 hrs.	96 hrs.	144 hrs.
				Trial 1				
) :	11.0	12.5	8.0	8.5	7.2	13.0	9.4	6.5
) :	16.0	14.0	6.0	5.8	13.0	22.0	16.0	12.0
) :	12.0	10.0	6.8	3.0	4.8	10.0	11.0	5.3
) :	4.5	8.5	4.5	3.5	14.0	22.0	20.0	14.0
) :	16.0	13.5	13.5	15.5	6.2	2.1	6.6	4.9
) :	6.0	13.0	20.0	11.5	15.0	6.9	5.7	3.1
) :	17.0	23.0	22.0	15.0	3.8	4.0	4.0	6.3
) :	17.0	22.0	22.8	15.0	13.3	3.8	5.3	3.1
) :	0.0	1.0	1.0	10.0	0.5	0.7	3.5	3.4
) :	11.8	13.5	17.0	1.5	11.0	4.6	8.2	23.0
				Trial 2				
) :	32.0	30.0	13.0	15.0	12.0	14.0	11.0	13.0
) :	31.0	23.0	16.0	11.0	15.0	18.0	23.0	14.0
) :	21.0	26.0	50.0	22.0	1.1	35.0	10.0	12.0
) :	31.0	20.0	24.0	8.0	13.0	17.0	21.0	8.4
) :	50.0	50.0	44.0	47.0	13.0	17.0	9.0	8.6
) :	42.0	45.0	32.0	34.0	24.0	14.0	9.5	7.1
) :	40.0	45.0	40.0	36.0	10.0	12.0	10.0	6.6
) :	52.0	44.0	42.0	35.0	31.0	11.0	6.7	6.9
) :	3.0	14.0	12.0	4.0	7.0	12.0	9.5	3.7
) :	31.0	32.0	23.0	19.0	26.0	17.0	9.3	13.0

after growth for 48, 72, 96, and 144 hours. Results of two trials are shown in Table 7.

As in the earlier trials the highest acid degree values were obtained at pH 4.5 and 5.5. The cell count also was highest at these pH levels. Observations made on the morphology of the cells in Trial 1 showed a predominance of short oval cells at pH 3.5 and 8.5 but long oval and slender cells at pH levels above 3.5 and below 8.5. A correlation between higher lipase production and long oval or slender cells was observed. Since pH levels of pH 4.5 and 5.5 resulted in the highest lipase production, further trials were conducted at this pH range.

Four 200 ml. quantities of nutrient broth at pH 4.5 and buffered with increasing concentrations of disodium phosphate-citric acid buffer were prepared and inoculated with 1 ml. quantities of growing cultures of M. lipolytica. The cultures were grown undistrubed for 72 hours, and examined for lipase activity and cell count. Results of two trials are shown in Table 8.

The results of the two trials showed higher lipase production at buffer concentrations of 0.450 and 0.675 m. than at 0.225 and 0.900 m. The population was less with higher buffer concentrations than in the presence of 0.225 m. buffer. Since these determinations were made late in the program, the higher buffer concentrations were not used in

Table 7. Additional Studies of the Influence of pH upon Lipase Production, Growth, and Shape of Cells of *M. lipolytica* (Results of Two Trials)

pH of medium	Acid degrees per ml. of culture		Count per ml. (in millions)		Predominant shape of Cells*
	Trial 1	Trial 2	Trial 1	Trial 2	
48-hour cultures					
3.5	3.0	2.0	0.8	0.7	Short oval
4.5	28.0	17.0	2.8	10.0	Long oval, slender
5.5	33.0	20.0	2.1	17.6	Long oval, slender
6.5	25.0	8.0	3.0	6.2	Long, short oval
7.5	23.0	7.0	3.6	2.3	Long, short oval
8.5	10.0	0.0	1.3	1.3	Short oval
72-hour cultures					
3.5	1.0	7.0	1.6	1.3	Short oval
4.5	40.5	44.0	3.7	33.0	Long oval, slender
5.5	35.0	29.0	3.5	14.3	Long oval, slender
6.5	34.0	18.0	2.2	13.4	Short oval, long slender
7.5	27.0	14.0	5.5	6.8	Short, long oval
8.5	15.0	6.0	3.1	1.8	Short oval
96-hour cultures					
3.5	12.0	7.0	4.6	2.4	Short oval
4.5	64.0	44.0	5.0	18.0	Long slender, filaments
5.5	43.0	23.0	3.5	15.0	Short slender, filaments
6.5	33.0	21.0	2.1	17.0	Long, short oval
7.5	24.0	18.0	5.7	19.0	Short, long oval
8.5	21.0	6.0	3.5	4.4	Short, long oval
144-hour cultures					
3.5	12.0	16.0	3.9	9.4	Short oval
4.5	52.0	43.0	5.5	20.0	Long oval, slender
5.5	34.0	24.0	3.8	7.7	Long, short oval
6.5	17.0	19.0	2.1	5.0	Short oval
7.5	15.0	7.0	6.3	9.6	Short oval
8.5	10.0	5.0	3.0	5.9	Short oval

* Examinations of first trial only.

Table 8. The Influence of Increasing Buffer Concentrations Upon the Production of Lipase and the Growth of *M. lipolytica* (pH 4.5)

Buffer concentration (molality)	pH after 72 hrs. growth		Acid degrees per ml. of culture		Count per ml. (in millions)	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
0.225	6.0	5.7	27	26	35	34
0.450	5.2	5.0	38	33	14	11
0.675	4.6	4.5	40	31	14	11
0.900	4.5	4.5	20	19	12	13

place of the 0.225 m. concentration which was used during most of the studies.

Growth temperature. In studying the effect of temperature upon lipase production, 200 ml. quantities of nutrient broth, buffered at pH 4.5, were inoculated and individual lots incubated at 21, 25, 30, and 36°C. Examinations for lipase activity and cell count were made after growth periods of 48, 72, 96, and 144 hours. A second trial was run in the same manner, except that the medium was adjusted to pH 5.5. Results of the two trials are shown in Table 9.

The results of both trials showed 30°C. as optimum for lipase production. Growth was most rapid and total population the greatest in cultures held at 25°C., and this temperature was second only to 30°C. in favoring lipase production. An incubation temperature of 36°C. was definitely inhibitory to both growth and lipase production, while lipase production was somewhat retarded at 21°C. but eventually reached fairly high levels.

Oxidation-reduction conditions. A series of four trials was conducted in which the yeast was grown under different oxidation-reduction conditions. In the first trial, 1.2 ml. of a 10 percent solution of methylene blue was added to 1200 ml. of nutrient broth, buffered at pH 5.5. The medium was divided into five equal portions, inoculated, and the portions treated variously. The control culture was shaken

Table 9. The Influence of Different Growth Temperatures on the Production of Lipase and the Growth of M. lipolytica

Growth temperature (°C.)	Acid degrees per ml. of culture: Trial 1 (pH 4.5)	Acid degrees per ml. of culture: Trial 2 (pH 5.5)	Count per ml. (in millions): Trial 1 (pH 4.5)	Count per ml. (in millions): Trial 2 (pH 5.5)
48-hour cultures				
21	1.0	4.0	1.2	0.9
25	18.0	15.5	4.4	1.9
30	29.0	39.0	2.8	1.5
36	1.0	1.0	0.2	0.2
72-hour cultures				
21	14.0	9.0	3.4	1.9
25	18.5	25.0	5.1	4.7
30	39.5	34.0	4.1	1.4
36	5.0	1.0	0.3	0.01
96-hour cultures				
21	30.5	17.0	3.3	1.6
25	17.0	25.0	6.7	6.6
30	44.0	46.0	4.1	0.9
36	11.0	1.0	0.3	0.01
144-hour cultures				
21	38.0	24.0	5.6	1.5
25	32.5	33.0	12.0	7.7
30	46.5	54.0	1.8	1.4
36	3.5	0.0	0.4	0.05

twice daily. Bacteria-free filtered air was bubbled through the second culture at a slow rate from a sintered glass gas-washing head. To each of three additional lots ascorbic acid, cysteine and thioglycolic acid, respectively, were added to the medium in quantities of 0.02 percent. These cultures were shaken twice daily. Examinations for lipase production, cell count, color changes of methylene blue, and shape of cells were made after growth for 48, 72, 96, and 144 hours. Results are shown in Table 10.

Aeration definitely restricted lipase production, while the addition of reducing compounds did not result in values significantly different from the control, although the latter medium retained its blue color because of the frequent shaking of the cultures. Aeration of the one culture resulted in a faster growth rate and short oval shape of the cells as compared with the smaller population and predominance of long oval cells in the control culture. Especially the culture in which cysteine had been incorporated, but also to some extent that to which thioglycolic acid had been added, showed somewhat greater lipase activity than the control at all times except at 144 hours. These two addenda also increased the population slightly.

In trial 2, three 200 ml. quantities of nutrient broth with methylene blue added as in Trial 1, and one without, and all buffered at pH 5.5 were inoculated and treated as

Table 10. The Influence of Oxidation-reduction Conditions Upon Lipase Production and Growth of M. lipolitica (Trial 1)

Variable factor	Acid degrees	Count per ml. (in millions)	Color of medium*	Predominant shape of cells
48-hour cultures				
Control	8.0	3.5	Blue	Long oval
Aeration	4.0	14.0	Blue	Short oval
Ascorbic acid	3.5	2.7	Yellowish-green	Short oval
Cysteine	14.0	5.8	Blue	Long oval
Thio glycolic acid	11.0	6.5	Yellow	Long oval
72-hour cultures				
Control	11.0	6.1	Blue	Long oval
Aeration	5.0	25.0	Blue	Short oval
Ascorbic acid	13.0	8.7	Greenish-yellow	Long oval
Cysteine	18.0	12.6	Green	Long oval
Thio glycolic acid	17.5	8.6	Yellow	Long oval
96-hour cultures				
Control	18.0	9.3	Blue	Long oval
Aeration	6.0	28.0	Blue	Short oval
Ascorbic acid	22.5	14.0	Greenish-yellow	Short, long slender
Cysteine	28.0	11.0	Green	Long oval
Thio glycolic acid	24.0	15.0	Yellow	Long oval
144-hour cultures				
Control	32.5	8.2	Blue	Long oval, slender
Aeration	5.0	31.0	Blue	Short oval
Ascorbic acid	28.0	11.0	Greenish-yellow	Short, long slender
Cysteine	35.0	11.0	Green	Long oval
Thio glycolic acid	31.5	14.0	Light green	Long oval

*Used as indicator of oxidation-reduction conditions.

follows: (a) The control containing no methylene blue was shaken twice daily. (b) To a second lot of basal medium 0.02 percent cysteine was added before inoculation and the culture was not aerated during incubation. (c) To a third lot of basal medium 0.02 percent cysteine was added after aerating for 48 hours; aeration was discontinued after the addition of cysteine. (d) After aerating for 96 hours, 0.02 percent cysteine was added to a fourth lot of basal medium, after which aeration was discontinued. Examinations for the production of lipase, cell count, and shape of cells were made after growth periods of 48, 72, 96, and 144 hours. Results are shown in Table 11.

Aeration again was demonstrated to be detrimental to the production of lipase. Aeration, followed by the addition of cysteine and cessation of aeration resulted in a gradual increase in lipase content of the culture. The shape of the cells also changed from short and oval to long and slender. Both cultures to which cysteine was added showed the same general behavior, although the shift to lipase producing form was greatest in the culture aerated for only 48 hours. The much higher lipase activity of the control culture, as compared to the culture to which cysteine was added without aeration may have been due to the absence of methylene blue in the control culture. Once more short oval cells were associated with the high counts and

Table 11. Additional Studies on the Influence of Oxidation-reduction Conditions Upon Lipase Production and Growth by M. lipolytica (Trial 2)

Variable factor	Acid degrees	Count per ml. (in millions)	Predominant shape of cells
48-hour cultures			
Control	18.5	4.2	Long oval
Cysteine at once	10.0	28.0	Long slender
Cysteine after 48 hr. aeration	9.5	22.0	Short oval
Cysteine after 96 hr. aeration	10.5	22.0	Short oval
72-hour cultures			
Control	33.0	4.5	Long oval, slender
Cysteine at once	14.5	25.0	Long slender
Cysteine after 48 hr. aeration	10.0	26.0	Short, long oval
Cysteine after 96 hr. aeration	8.0	40.0	Short oval
96-hour cultures			
Control	45.0	11.0	Long oval, slender
Cysteine at once	23.0	25.0	Long slender
Cysteine after 48 hr. aeration	16.0	16.0	Short, long slender
Cysteine after 96 hr. aeration	4.0	79.0	Short oval
144-hour cultures			
Control	41.0	4.9	Long oval, slender
Cysteine at once	19.0	18.0	Long slender
Cysteine after 48 hr. aeration	18.5	15.0	Long slender
Cysteine after 96 hr. aeration	15.0	19.0	Short, long oval

low acid degree values resulting from aeration of cultures.

In no case did addition of cysteine result in a significant increase in lipase production, although it may have assisted in bringing about favorable conditions for lipase production after the cessation of aeration. In a further study of the influence of aeration and cysteine upon lipase production, trials 3 and 4 were run, each performed with four 200 ml. portions of nutrient broth, buffered at pH 4.5, which were treated as follows: (a) Shaken every 12 hours; (b) Shaken every 48 hours; (c) 0.02 percent cysteine in medium and shaken every 12 hours; (d) 0.02 percent cysteine in medium and shaken every 48 hours. Examinations for lipase production, population and shape of cells were made after 48, 96, and 144 hours of growth. Results are shown in Table 12.

Shaking of cultures every 12 hours retarded the rate of lipase production in the younger cultures, as higher acid degree values were obtained from cultures b and d, which were shaken only every 48 hours, than from cultures a and c, shaken every 12 hours. Acid degree values after 144 hours were in favor of the cultures shaken every 12 hours. The difference in lipase activity in cultures with and without added cysteine, but otherwise given similar treatment, was not significant except at 96 hours in Trial 3. The cell count per milliliter could not be correlated with lipase

Table 12. The Influence of Shaking and Addition of Cysteine on Lipase Production and Growth of M. lipolytica

Variable factor	Acid Degrees		Count (in millions)		Predominant shape of cells	
	Trial 3	Trial 4	Trial 3	Trial 4	Trial 3	Trial 4
48-hour cultures						
a. Shaken every 12 hrs.	15.0	21.5	6.8	13.0	Short, long oval	Long oval
b. Shaken every 48 hrs.	66.0	38.0	30.0	28.0	Long slender	Long slender
c. Cysteine + (a)	16.0	19.0	9.8	8.5	Short, long oval	Long oval
d. Cysteine + (b)	32.0	34.0	14.0	12.0	Long oval, slender	Long oval, slender
96-hour cultures						
a. Shaken every 12 hrs.	34.0	26.0	11.0	11.0	Long oval, slender	Long, short slender
b. Shaken every 48 hrs.	35.5	22.0	30.0	28.0	Long slender	Long, short, slender
c. Cysteine + (a)	63.0	26.0	8.9	12.0	Long slender	Long, short, slender
d. Cysteine + (b)	73.0	30.0	14.0	10.0	Long slender	Long, short, slender
144-hour cultures						
a. Shaken every 12 hrs.	43.0	21.0	9.0	6.3	Long oval, slender	Long, short slender
b. Shaken every 48 hrs.	22.0	14.0	38.0	47.0	Long slender	Long, short oval
c. Cysteine + (a)	44.0	25.0	12.0	17.0	Long slender	Long, short slender
d. Cysteine + (b)	36.5	21.0	19.0	7.0	Long slender	Long, short slender

production, although the greatest production of the enzyme in the younger cultures occurred in those cultures containing the most organisms. However, the shape of the cells again was found to correlate with the lipase activity, long oval or slender cells being associated with high acid degree values.

Factors influencing the activity of M. lipolytica lipase

pH of substrate. In a preliminary trial the reaction of the substrate-emulsion used for determining the activity of the M. lipolytica lipase was adjusted at intervals of 0.5 pH units over the range from pH 4.0 to pH 8.0. Disodium phosphate (0.2 m.) and citric acid (0.1 m.) were adjusted to desired pH levels and added to equal volumes of a 10 percent butterfat-agar emulsion. The remaining steps were those described under "Methods". Results of this trial are shown in Table 13.

The results of this preliminary trial showed that greatest hydrolysis of butterfat was obtained in the range pH 6.0 - 7.0.

Two additional trials were run over a narrower pH range, namely pH 5.8 - 7.2. The results obtained are shown in Table 14.

The results obtained in the two additional trials showed that pH 6.2 - 6.5 was most suitable for hydrolysis of butterfat by M. lipolytica lipase under the method employed

Table 13. The Influence of pH Upon M. lipolytica Lipase Activity

pH of substrate	Acid degrees per ml. of enzyme preparation
4.0	16
4.5	22
5.0	24
5.5	26
6.0	32
6.5	55
7.0	31
7.5	15
8.0	12

Table 14. Further Determinations of the Influence of pH Upon M. lipolytica Lipase Activity

pH of substrate	Acid degrees per ml. of enzyme preparation	
	Trial 1	Trial 2
5.8	30.0	30.0
6.0	36.5	32.0
6.2	40.0	40.0
6.4	34.5	-
6.5	-	55.0
6.6	25.5	-
6.7	-	37.0
6.8	23.5	-
7.0	22.5	31.0
7.2	13.5	-
7.4	12.0	-
7.5	-	15.0

and the reaction of the butterfat emulsion for detection of lipolysis was adjusted to pH 6.5 in subsequent studies. The ability of this enzyme system to cause considerable lipolysis in the pH range from 5.0 to 6.0 indicated the possibility of its use in the somewhat acid curd of cheese.

In five trials in which pasteurized homogenized milk was substituted for butterfat-agar emulsion as substrate, acid degree values ranging between 47 and 87 were obtained, causing a lowering in pH of milk up to 0.9 of a pH unit. These results served as a good indication of M. lipolytica lipase activity in milk.

Temperature and time. In trial 1, M. lipolytica lipase activity was studied at temperatures of 30, 37, 45, and 52°C. Determinations were made at 8-hour intervals up to 48 hours total reaction time. The results of this trial are shown in Table 15.

Incubation temperatures of 37 and 45°C. brought about more rapid lipase activity in the first 8-hour period than was the case with temperatures of 30 and 52°C. Highest acid degree values were obtained in the emulsion held at 37°C. Early inactivation of the enzyme took place at 52°C. Thus this temperature was omitted from further trials. The action at 30°C. was somewhat slower than at 37 and 45°C., but persisted longer at a uniform rate, as shown by the continued increments of free acidity.

Table 15. The Influence of Temperature and Time Upon the Activity of M. lipolytica Lipase

Reaction time in hours	Acid degrees after various incubation periods at temperatures of			
	30°C.	37°C.	45°C.	52°C.
8	8.0	17.0	17.0	8.0
16	14.0	20.0	20.0	9.0
24	17.0	29.0	20.0	10.0
32	20.0	35.0	26.0	14.0
40	25.0	37.0	25.0	9.0
48	28.0	39.0	26.0	10.0

Table 16. Further Studies on the Influence of Temperature and Time Upon M. lipolytica Lipase Activity (Average of 2 trials)

Reaction time in hours	Acid degrees after various incubation periods at temperatures of			
	28°C.	33°C.	37°C.	43°C.
6	11.0	10.5	9.0	12.0
18	20.0	19.5	14.0	15.0
30	22.5	27.5	19.5	16.5
42	35.5	32.5	28.5	18.0
54	43.0	41.0	31.0	18.0
66	45.0	43.0	30.0	17.0
78	49.0	48.5	31.5	20.0
102	62.5	63.0	34.0	20.5
126	70.0	72.5	36.0	23.0
150	74.0	73.5	33.0	19.5

Two further trials were conducted with reaction temperatures of 28, 33, 37, and 43°C. Titrations were made at incubation periods up to 150 hours. Results are shown in Table 16. The results of these trials showed temperatures of 28 and 33°C. to be more favorable for lipase activity than were 37 and 43°C. The effect of temperature upon lipase activity became more pronounced with increased holding time. The action of the enzyme at 37 and 43°C. after 54 hours holding time was practically at a stand still. This was not the case at 28 and 33°C., for a continuous increase in acid degree values took place even though the rate decreased somewhat with increased holding time.

An additional trial was conducted using temperatures of 10, 21, 28, and 33°C. Titrations were made at increasing intervals up to 810 hours in case of 10 and 21°C., and up to 330 hours with 28 and 33°C. holding temperatures. The results are shown in Table 17.

The rates of lipase activity at temperatures of 21, 28, and 33°C. were similar, while at 10°C. lipase activity was somewhat retarded. The acid degree values obtained at 810 hours incubation time were 40 and 46 at 10 and 21°C., respectively, the difference not being considered of great significance.

In summarizing the results of this series of trials, temperatures of 33°C. and below were found to be more

Table 17. The Influence of Lower Temperatures and Prolonged Holding Time upon the Activity of M. lipolytica lipase

Time in hours	Acid degree values after incubation at temperatures of: 10°C.	21°C.	28°C.	33°C.
18	8	12	11	16
42	-	-	19	22
66	-	22	23	23
90	13	-	21	23
114	-	27	26	25
138	-	-	28	30
162	20	33	29	26
186	-	-	28	27
210	-	32	30	27
234	19	-	29	32
282	-	-	33	35
306	27	40	-	-
330	-	-	29	34
354	-	37	-	-
402	-	37	-	-
450	28	39	-	-
498	-	46	-	-
594	28	39	-	-
666	35	-	-	-
738	-	-	-	-
810	40	46	-	-

desirable for M. lipolytica lipase activity if incubation periods exceeded 48 hours. Incubation at 37°C. was favorable to enzyme activity at periods of 48 hours or less, while temperatures of 43°C. and above proved to be inactivating when the incubation period extended more than 8 or 12 hours.

Amounts of lipase added. Two trials were run with the amount of enzyme used in the individual determinations as the variable. A lyophilized culture concentrate was used in quantities of 0.01, 0.03, 0.05, 0.1, 0.2, 0.4, and 0.8 ml. The regular determination procedure was followed, using a 48-hour incubation period at 37°C. Results are shown in Table 18.

Higher acid degree values were obtained with the addition of larger amounts of enzyme. However, doubling the amount of lipase did not double the determined acid degree values. The calculated acid degree values on the one milliliter basis showed increasing values with lower lipase concentrations present in the sample, which indicated greater total lipase activity per unit of lipase over the 48-hour period than in other samples containing higher lipase concentrations. A close correlation existed in the acid degree values obtained in the two trials with the same enzyme concentrate, indicating a good degree of reproducibility by this method.

Storage condition and time. A cell-free filtrate was prepared from a 3-day old culture of M. lipolytica by ultra-

Table 18. The Influence of Varying Amounts of M. lipolytica Lipase Upon Lipase Activity as Determined by the Normal Procedure

Amount of concentrate used (ml.)	Acid degrees as Determined per increment used: Trial 1	Trial 2	Acid degrees as Calculated per ml. of concentrate used: Trial 1	Trial 2
0.01	5	5	500	500
0.03	6	8	200	267
0.05	9	9	180	180
0.1	15	15	150	150
0.2	19	21	90	105
0.4	33	32	82	80
0.8	42	42	52	52

Table 19. The Influence of Storage Temperature, Time, and the Presence of Cysteine Upon the Lipase Activity of M. lipolytica

Storage condition	Acid degrees after Storage time (in days) Trial 1: 0	4	106	Storage time (in days) Trial 2: 0	4	96
3 - 5°C.	25	22.5	22	32	26	28
23 - 25°C.	25	17.5	13	32	26	16
23 - 25°C. cysteine	25	21.0	14	32	18	16

filtration through ultra-fine sintered glass. Portions of the filtrate were stored in screw-cap test tubes, one at 3 - 5°C. and two at room temperature (23 - 25°C.); one of the room-temperature tubes contained 0.002 percent added cysteine. The samples were examined for lipase activity at 0, 4, and 106 days. A second trial was run in a similar manner and examinations made after 0, 4, and 96 days storage. Results of the two trials are shown in Table 19.

The results of both trials showed that the lipase activity tended to decrease upon storage. This decrease was more pronounced at 23 - 35°C. than at 3 - 5°C. The presence of 0.002 percent cysteine did not prevent the inactivation of lipase during storage. A low storage temperature was found the most important factor in the preservation of lipase activity.

Concentration and purification of M. lipolytica lipase

Concentration by lyophilizing. Three trials were conducted using 3-day old nutrient broth cultures of M. lipolytica. Lyophilizing was done as described under "Methods". The results of these trials are shown in Table 20.

Concentration of M. lipolytica cultures by lyophilizing, either partially or to complete dryness, resulted in concentration of lipase as well. However, lyophilized portions, equivalent to 1 ml. of the original culture, yielded acid

Table 20. The Effect of Lyophilizing Treatment of M. lipolytica cultures Upon its Lipase Activity

Item studied	Trial number		
	1*	2*	3
A.D. per ml. of original culture**	36	70	54
Original weight of culture**	200 gm.	183 gm.	190 gm.
Weight of culture after lyophilizing	50 gm.	31 gm.	12 gm.
A.D. of 1 ml. equiv. of orig. culture	19	24	26
A.D. ratio of orig. to lyophilizing culture	2:1	3:1	2:1

* Incompletely dried.
** Calculated as 1 ml. = 1 gm.
A.D.=Acid degree.

degree values about one half of the values of the original culture in two cases, and only one third of the original value in one case. Continued lyophilizing up to the change from liquid to solid state of the culture resulted in a sticky, gluey mass which was difficult to handle, but which dissolved readily in distilled water or in phosphate buffer solution, and was possessed of high enzyme activity per unit mass.

Concentration by foaming. Two trials were run first to determine the most suitable pH level for the concentration of M. lipolytica lipase by this procedure. One liter of 3-day old culture was centrifuged and 700 ml. of the supernatant liquid divided into 100 ml. portions which then were adjusted to pH levels between pH 4.0 - 7.0 by means of concentrated hydrochloric acid or sodium hydroxide. Compressed carbon dioxide was used as foaming agent, and the first 10 ml. quantity of condensed foam was collected from each 100 ml. portion of enzyme preparation. Lipase activity measurements were made on the resulting 10 and 90 ml. portions. Results of the two trials are shown in Table 21.

While there was a measureable increase in lipase concentration in the liquid foam portions, as compared with the lipase concentration of the original supernatant, yet the acid degree values obtained did not point definitely to any one pH level as the most suitable one for the

Table 21. The Influence of pH Upon the Concentration of M. lipolytica Lipase by the Foam Adsorption Method

pH of supernatant	Acid degrees per ml. in the First 10 ml. of liquid foam		Acid degrees per ml. in the Residual 90 ml. of supernatant	
	Trial 1	Trial 2	Trial 1	Trial 2
Original (5.2)	31	44	31	44
4.0	43	64	1	3
4.5	31	57	7	4
5.0	39	55	12	10
5.5	44	37	7	3
6.0	41	64	6	6
6.5	45	50	5	3
7.0	41	44	4	6

Table 22. The Influence of Repeated Foaming Upon the Concentration of Lipase Produced by M. lipolytica

Number of fractionations	Volume of fraction (ml.)	Acid degrees per ml. of Liquid foam		Acid degrees per ml. of Residue	
		Trial 1	Trial 2	Trial 1	Trial 2
Original	800	27	24	27	24
1	400	20	24	3	4
2	200	26	34	1	3
3	100	40	40	2	0
4	50	50	50	0	4
5	25	67	60	0	1

concentration of lipase produced by M. lipolytica. Examination of the acid degree values obtained at the different pH levels showed that pH 4.0 was high in the lipase activity of the liquid foam portion and lowest in the residual portions, although the margin was small. This pH level, therefore, was selected for further work on the foam-adsorption method and the concentration of lipase produced by M. lipolytica.

In further studies on the foam-adsorption procedure for concentration of lipase 3-day old cultures were centrifuged and the supernatant adjusted to pH 4.0 with concentrated hydrochloric acid. Quantities of 800 ml. of supernatant were fractionated by foaming to one-half volume and the process repeated five times. Lipase activity measurements were made at each step, both on the liquid foam and on the residue portion. Results of two trials are shown in Table 22.

While the first fractionation did not result in an increase in lipase concentration, as judged by the acid degree values obtained, further fractionations resulted in increases in lipase concentration. Either the removal of the lipase by 50 percent fractionation was practically complete, or the lipase in the residue was destroyed, since the residues showed little or no lipase activity. The increase in acid degree values in the two trials was approximately 250 percent in each case. The lipase remaining in the final foam fraction represented about 8 per-

cent of the initial lipase activity, according to the figures as presented.

Concentration by salting out. Preliminary trials were conducted in test tube quantities with 3-day old cultures of M. lipolytica, using magnesium sulfate, ammonium sulfate and sodium chloride as precipitating agents. Precipitation of a lipase-active substance occurred only in saturated solutions of ammonium sulfate. Thus ammonium sulfate was used in further trials with larger quantities of cultures.

Supernatant of 3-day old cultures in 400 ml. quantity was placed into a one liter separatory funnel, ammonium sulfate added to the saturation point and followed by the addition of 30 percent by volume of 95 percent ethyl alcohol. This procedure resulted in the collection of the lipase-active precipitate at the water-alcohol interface, and the flocculent precipitate then was collected and dried as described under "Methods". Table 23 shows the results of three trials.

Although only a small portion of the original total lipase was recovered in form of a white precipitate, this precipitate represented concentrations of from 8 to 20 times the original, based on acid degree values obtained from the original supernatant and the precipitate.

Further work was done to compare the effect of different

Table 23. Concentration of Lipase by the Salting-out Procedure with Ammonium Sulfate and Ethyl Alcohol

Trial number		Weight or volume of total	Acid degrees per ml. or gm.	Recovery of enzyme* (%)
I	Liquid	400	14.0	-
	Dry	0.25	280.0*	1.25
II	Liquid	400	25.0	-
	Dry	0.20	200.0*	0.4
III	Liquid	400	23.0	-
	Dry	0.18	200.0*	0.39

*Calculated from increment used.

Table 24. The Influence of Temperature During Precipitation Time Upon the Lipase Activity of the Precipitate

Items studied	Trial 1		Trial 2	
	6°C.	24°C.	6°C.	24°C.
A.D. per ml. of original supernatant	25	25	23	23
Total weight of precipitate in gm.	0.2	0.2	0.16	0.18
Acid degrees per 0.03 gm. of ppt.	21	6	44	6
Calculated A.D. per total ppt.	140	40	300	36
Calculated percent recovery of original total lipase activity	1.4	0.4	3.0	0.36

A.D.=Acid degree.

temperatures during the process of precipitation upon the yield and lipase activity of the precipitate. The same method was followed as before, treating 400 ml. of supernatant at room temperature (24° C.) while an equal quantity of the same supernatant was cooled in ice water to 6°C. and kept at this temperature until the drying of the precipitate. Results of two trials are shown in Table 24.

The results of the two trials were much in favor of the lower temperature. The lipase activities of the precipitates obtained at 6°C. were 250 and 730 percent greater than the activities of the corresponding precipitates as compared with the lipase activity prepared at room temperature. Further study of possible alternatives in the procedure might result in greater percentage recovery, as very little exploratory work was done in this phase of the study.

The influence of the addition of various amounts of *M. lipolytica* lipase upon the ripening of blue cheese made from pasteurized homogenized milk

Three series of three lots each and one series of four lots of cheese were made with pasteurized homogenized milk by the procedure outlined under "Methods". In series 1 and 2 the clear supernatant liquid obtained by centrifuging 3-day cultures was used while in series 3 and 4 cell-free filtrates from the cultures were added to the milk in various

Table 25. The Influence of the Addition of Various Amounts of M. lipolytica Lipase Upon the Volatile Acidity, Flavor, and Mold Growth of Blue Cheese

Lot no.	Amount of lipase added*	Total volatile acidity		Flavor score		Defect score		Mold score		Defect comments on 12 week old cheese
		4 wks.	12 wks.	4 wks.	12 wks.	4 wks.	12 wks.	4 wks.	12 wks.	
11	none	6.0	18	**	-	-	-	-	-	Flat, slightly unclean
12	100	6.6	18	-	-	-	-	-	-	Flat, slightly unclean
13	200	7.0	16	-	-	-	-	-	-	Sl. unclean, sl. bitter
21	none	5.4	13	-	-	-	-	-	-	Flat, sl. unclean
22	300	9.6	22	-	-	-	-	-	-	Sl. unclean, sl. soapy
23	800	18	65	-	-	-	-	-	-	Too high in acid, soapy
31	none	7.0	13	3.0	4.0	4.0	3.5	3.5	4.0	Sour, flat
32	660	26	50	7.0	7.5	6.5	7.0	4.0	7.0	Sl. sour, sl. soapy, sl. sharp
33	1300	39	85	6.5	6.0	5.5	3.0	3.5	5.0	Excessively sharp and soapy
41	none	9.0	18	3.5	4.0	6.0	3.5	7.5	7.5	Sl. nutty, sl. fermented
42	300	15	38	6.5	5.0	6.0	4.0	3.5	7.0	Sl. nutty, sl. unclean
43	600	21	60	8.0	7.5	8.0	7.0	5.5	4.5	Sl. sharp, sl. soapy
44	900	26	72	7.0	5.5	5.5	3.5	3.5	6.0	Excessively sharp and soapy

* Calculated as total activity (ml. of preparation x acid degree value per ml.) added to 115 lbs. of milk.

** No numerical scores given (for Lots 11, 12, 13, 21, 22, and 23).

amounts. A control cheese without added lipase was made in each series. Data concerning the manufacturing operation and the chemical analyses of the cheeses are shown in the Appendix, while the total volatile acidity values, flavor, defect, and mold scores of the cheese at 4 and 12 weeks are presented in Table 25.

The results obtained with the four series of cheese showed that the addition of M. lipolytica lipase to the milk resulted in increases in the total volatile acidity of the cheese proportional to the amount of lipase added. In all four cases the control cheese had the smallest amounts of total volatile acidity in their respective series, and were lacking in the desired ketone flavor. Cheeses made with the higher levels of lipase additions resulted in excessively sharp and soapy tastes (Lot 23, 33, 44). Best results were obtained with cheeses 32 and 43, although they also were criticized for being slightly soapy and slightly sharp. There was no indication that mold growth was affected by the different amounts of total volatile acids present in the individual cheeses. No evidence was obtained that any of the defects found in the cheese made with the added lipase were attributable to the enzyme preparation used. In general the defect scores were somewhat improved by the addition of the lipase preparations, even though excessive degradation of the fat was considered and scored as a flavor defect. The

data suggest that almost any desired level of fat hydrolysis may be obtained by use of enzyme preparations from M. lipolytica in pasteurized milk to be made into blue cheese.

DISCUSSION

The part of the study concerned with the production of lipase by M. lipolytica revealed that the composition of the medium and its reaction play important roles in lipase production. Other factors of equal importance were growth temperature of the organism and the oxygen level of the medium. Conditions favorable for rapid growth of the organism were found undesirable for the production of lipase. The presence of glucose in the medium resulted in more prolific growth and less lipase production than if glucose was omitted. A similar observation with P. roqueforti was made by Thibodeau (1940). Golding (1934) worked with the same type of mold and also reported that glucose accelerated the growth of P. roqueforti considerably. He also observed the shifting of the reaction of the medium towards the acid side if glucose was present, an observation also made in the present study with M. lipolytica. The fact that the buffered growth medium with 1 percent glucose added increased in acidity, while with no glucose added the acidity decreased, led to the conclusion that the organism utilized the glucose at a rather rapid rate. This utilization, however, resulted in increase of population rather than in lipase formation. Only in a relatively glucose-free nutrient broth did maximum lipase production take place. Apparently the utilization of organic nitrogenous compounds, such as peptone,

is required to produce considerable amounts of lipase under the conditions of this study. The slenderness of cells in cultures high in lipase was observed repeatedly, contrasting with the short, oval cells characteristic of the cultures which produced little lipase.

Other growth conditions being equal, the hydrogen ion concentration of the medium also affected the production of lipase by M. lipolytica. This effect varied in magnitude with the kinds of buffers employed. Disodium phosphate and monopotassium phosphate were comparable in their effect to disodium phosphate and citric acid, at pH 7.5 and pH 5.5. Sodium acetate and acetic acid, however, were less suited for lipase production, especially at a concentration of 0.225 m and pH 5.5. A pH of 4.5 to 5.5 with phosphate buffers was most desirable for the production of lipase by M. lipolytica. These pH levels retarded the growth of the organism as compared with higher levels, such as pH 6.5 and pH 7.5, but resulted in greater lipase production per cell than at lower or higher pH levels. Here again, as in the case of the composition of the medium, somewhat surpressed growth resulted in maximum accumulation of lipase in the medium. Buffer concentrations of from 0.45 to 0.9 m. resulted in extremely slow growth, but the lipase production reached a higher level than at lower buffer concentrations at the same pH even though the lower buffer concentrations were able to maintain the reaction of the medium.

A growth temperature of 30° C., while producing less

growth than at 25° C., resulted in the accumulation of the largest amounts of lipase in the medium. Higher and lower temperatures gave less lipase activity than did 25 and 30° C. It is of interest to note that Weisbrodt (1927) observed P. roqueforti also produced most lipase at 30° C.

The addition of reducing substances to the growth medium did not result in significant increases in lipase production. An oxygen level permitting a slight reduction of methylene blue in the medium was found most satisfactory for lipase production. The E_h range at which methylene blue changes color at pH 4.5 is from +200 to 0 mv. (Hewitt, 1930). Rosenfeld (1946) found that obligatory anaerobic sulfate reducing bacteria displayed best lipolysis at E_h levels of -100 to -400 mv. Aerated cultures of M. lipolytica produced extremely high populations, but little lipase was accumulated in the medium. Whether or not lipase was produced in all instances but was utilized by the rapidly growing cells or destroyed due to the greater amount of oxygen present in the aerated cultures is not known. The fact that even twice-a-day shaking of cultures sufficed to cause lower lipase activity indicated that the oxidation-reduction conditions of the medium were of greater significance than they were considered to be in the early trials. The lack of this knowledge undoubtedly resulted in lower lipase activity in the trials where twice-a-day shaking was practiced. However, since all cultures in the same trial were treated identically, the comparison value of each trial remains unaffected.

Unquestionably further work on the factors influencing lipase production by M. lipolytica is essential in order to obtain a more complete picture of the interaction of the different factors and their effect upon the production of the enzyme.

Activity of lipase upon fat was detectable at pH 4.0, the activity increasing to the maximum at pH 6.2-6.5 and decreasing more rapidly up to pH 8.0. This observation was of much interest, since the ripening of blue cheese falls within the range of pH 4.7-6.5 (Coulter, Combs and George, 1938), a range in which this enzyme is relatively active. Trials conducted to determine the activity of this enzyme in homogenized milk verified this observation, which also was corroborated later by the changes produced in cheese in which this enzyme had been incorporated. The observation of Fouts (1939) that M. lipolytica produced rancidity in cream of relatively high acidity correlated with the results obtained in the present study.

Since the object of this problem was to learn as much as possible about the suitability of the enzyme system for use in making blue cheese from pasteurized milk, detailed study of the kinetics of the enzyme was not undertaken. In considering the effect of temperature and time upon the activity of the lipase of M. lipolytica, the observation was made that temperatures from 37 to 48° C. resulted in higher acid degree values early in the incubation periods but the enzyme activity at these temperatures decreased rapidly with time. Heat-inactivation of pancreatic lipase at temperatures above 40° C. was

observed by Sizer and Josephson (1942), and it is possible that the lipase considered in the present study was affected in a similar manner.

Additions of different amounts of enzyme to the same amount of substrate demonstrated greater calculated activity per unit of enzyme-containing material when the amount of such material added was small. When the enzyme activity per unit of material was considerable, as frequently was the case during the studies on methods of concentration, the results may indicate a level of activity somewhat below that which might be calculated from the use of smaller increments. Further work would be necessary in order to obtain a more complete knowledge of this situation.

The results with stored cell-free enzyme preparations showed the preservative effect of low storage temperature upon the enzyme activity. The preservative effect of low temperature storage upon enzyme activity also was observed by Kunitz and McDonald (1946) in their work with hexokinase. Storage at temperatures above 5° C. resulted in a gradual decrease of enzyme activity. They observed that inactivation was accompanied by denaturation of the enzyme-protein.

Of the three methods used for concentration of the lipase, the lyophilizing procedure resulted in the least destruction of the enzyme. However, it was difficult to handle the sticky mass consisting of yeast cells and the solid ingredients of the medium, including the buffer salt, and thus a more suitable

method was desired. The foaming method developed by Ostwald and Siehr (1937) had proven successful for separation of proteinaceous material from its liquid phase. While this method permitted the separation of M. lipolytica lipase from the bulk of the supernatant liquid, the losses of enzyme encountered during the process were great. The first fractionation did not show an increase in acid degree values. The same observation also was made by Ostwald and Mischke (1940) with diastase and was attributed by them to denaturation of protein during the foaming process. The effect of room temperature, which was not considered during this study, but as was observed later on the salting-out procedure, may be very significant. Here again the low temperature procedure followed by Kunitz and McDonald (1946) in their concentration by salting-out may be considered of interest.

The salting-out of M. lipolytica lipase with saturated ammonium sulfate was the only successful concentration procedure of this type tried. The use of fully saturated magnesium sulfate or sodium chloride proved unsuccessful for the precipitation of this enzyme. The salting-out with ammonium sulfate was hastened by the addition of 30 percent of 95 percent ethyl alcohol, which amount was found to be most useful in this procedure. Additions of smaller amounts of alcohol failed to produce separate layers of alcohol and water, while higher concentrations were considered undesirable due to the possible detrimental effect of the alcohol upon the enzyme. The fact

that the enzyme-active substance could be precipitated in a manner similar to the one used for pancreatic lipase by Glick and King (1933) seemed to indicate that M. lipolytica lipase might be regarded as an albumin. No attempt was made to crystallize or to study the precipitate beyond this point.

The addition of M. lipolytica lipase to pasteurized homogenized milk which then was made into blue cheese brought about the desired hydrolysis of the fat into glycerol and free fatty acids. Good correlation was found to exist between the amount of enzyme added and the total volatile fatty acid values obtained. The ripening conditions of the cheese, such as moisture, pH and temperature, all were favorable for the action of the enzyme. The study of M. lipolytica by Long (1936) had shown that this organism was capable of splitting a large number of pure triglycerides, including triolein. P. roqueforti on the other hand, as shown by Jensen (1941), was most actively lipolytic on the triglycerides from tributyrin up to and including tricaprin. According to Hammer and Bryant (1937), under unfavorable growth conditions this mold changes caprylic acid into methyl-n-amyl ketone, the latter compound being of considerable importance as a contributor to the flavor of blue cheese. The liberation by the enzyme of M. lipolytica of fatty acids which are not utilized or changed by the mold may be regarded as responsible for the soapy taste of the cheese. If this is the case, then it would be desirable to add the smallest quantity of enzyme capable of liberating the optimum amount of free fatty acids required for ketone formation by the mold. This

seems possible with a standardized enzyme preparation. No defects in the cheese could be traced to the enzyme added, except excessively sharp and soapy tastes produced in lots containing relatively high levels of lipase.

With the present knowledge of the factors responsible for lipase production of M. lipolytica, it undoubtedly would be possible to grow the organism in large quantities and to obtain high enzyme accumulation in the medium. The unconcentrated cell-free preparation is relatively stable at low temperatures and could be held in the plant for some time for use as needed. Further work unquestionably would result in greater recovery of enzyme during the process of concentration, and some level of purification also may be attained, but probably neither of these would be essential or possibly be desirable for the product to be used in the manufacture of blue cheese from pasteurized milk.

The use of this lipase preparation in blue cheese in a limited number of trials has shown its usefulness and suitability as a substitute for milk lipase inactivated by pasteurization. The flavor scores and volatile acidity values of the cheese containing proper amounts of the lipase from M. lipolytica have been appreciably higher than those of the control cheese.

The suitability of this enzyme preparation for manufacture of types of cheese other than blue in which some degree of lipolysis is desired has not been investigated, but the product offers considerable possibilities for such purposes, and studies to determine its suitability in this connection unquestionably should be made.

SUMMARY AND CONCLUSIONS

Mycotorula lipolytica was selected for this study since it possessed strong lipolytic and weak oxidative properties, and produced considerably more lipase in nutrient broth than either Achr. lipolyticum or Ps. fragi.

Of the five growth media tried, nutrient broth containing 0.05 percent glucose was most suitable for lipase production by M. lipolytica. Defined synthetic media and skim milk or homogenized milk proved much less satisfactory.

Lipase production by M. lipolytica was increased further by the addition of 0.225 m disodium phosphate plus citric acid to the nutrient broth with a reaction of pH 4.5-5.5. An equal addition of disodium phosphate plus monopotassium phosphate gave similar high results. A sodium acetate plus acetic acid buffer was unsatisfactory.

Growth at 30° C. was optimum for the production of lipase by M. lipolytica.

The accumulation of lipase in the medium was favored by growing the culture without shaking.

In general short oval cells and low lipase activity were associated with conditions favorable for rapid growth, while long oval and slender cells and high lipase activity were associated with conditions slightly less favorable for growth.

Lipase activity on butterfat was demonstrated over a range of reaction from pH 4.0 to pH 8.0, with pH 6.2-6.5 as optimum.

Lipase activity on butterfat was demonstrated at temperatures from 10 to 52° C.; temperatures of 37° C. and above had an inactivating effect upon the enzyme, the effect being more pronounced with the higher temperatures.

A storage temperature of 5° C. was much superior to 25° C. for preserving the enzyme in cell-free preparations, activity being maintained at a high level for at least 3 months when storage was at the lower temperature in closed containers.

Lyophilized enzyme preparations retained about half their original total lipase activity.

Considerable concentration of lipase by foaming was demonstrated; high losses of enzyme occurred during the process.

Salting-out of lipase with saturated ammonium sulfate plus 30 percent ethyl alcohol resulted in a white precipitate high in lipase activity.

The beneficial effect of M. lipolytica lipase upon the ripening of blue cheese made from pasteurized homogenized milk was demonstrated. The applicability of this enzyme in the production of blue cheese from pasteurized milk has been discussed.

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APPENDIX

Table 26. Data on Cheese Making and Cheese Analysis

Lot number	Milk acidity	* Whey acidity	Fat in milk	** Fat in cheese	** Moisture in cheese	** Salt in cheese	$CaCl_2$ added to milk
	%	%	%	%	%	%	%
11	0.17	0.16	3.8	28.1	44.4	3.8	-
12	0.17	0.16	3.8	29.5	42.5	4.3	-
13	0.17	0.16	3.8	30.7	41.2	4.2	-
21	0.17	0.18	3.8	32.0	40.1	4.3	-
22	0.17	0.20	3.8	32.8	39.4	4.4	-
23	0.17	0.20	3.8	32.2	38.8	4.8	-
31	0.16	0.23	3.5	26.6	48.8	3.7	0.015
32	0.16	0.23	3.5	27.6	49.5	3.0	0.015
33	0.16	0.23	3.5	24.8	50.4	3.2	0.015
41	0.16	0.16	3.5	27.5	47.6	4.3	0.015
42	0.16	0.16	3.5	27.0	49.0	4.9	0.015
43	0.16	0.16	3.5	26.3	48.9	5.8	0.015
44	0.16	0.16	3.5	25.8	49.8	5.1	0.015

* Acidity at time of dipping.
** Analysis of 4-week-old cheese.