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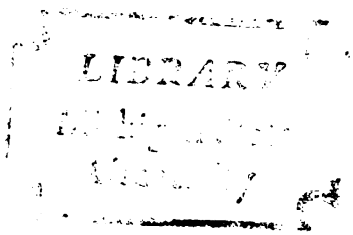

This is to certify that the

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Factors Affecting Production, Stability and Activity of Glycerol Ester Hydrolase of Streptococcus thermophilus

presented by

Jose Mauro de Moraes

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FACTORS AFFECTING PRODUCTION, STABILITY AND ACTIVITY OF GLYCEROL ESTER HYDROLASE OF *Streptococcus thermophilus*

By

Jose Mauro DeMoraes

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ABSTRACT

FACTORS AFFECTING PRODUCTION, STABILITY AND ACTIVITY OF GLYCEROL ESTER HYDROLASE OF *Streptococcus thermophilus*

By

Jose Mauro DeMoraes

Thirty two cultures of *Streptococcus thermophilus* were studied. The cells were harvested by centrifugation, washed and disrupted by pressure. All the strains except two showed GEH activity. The culture supernatant showed no activity. A selected strain ST-9, was studied for GEH production, activity and stability. Optimum temperature for GEH production was 44°C. Production was inhibited by butter oil (29%), milk (41%) and casein (12%) and stimulated by soybean oil (39%), cream (27%) and corn oil (21%). The enzyme was stable at 37°C and 45°C, partially inactivated by pasteurization and completely inactivated by boiling and sterilization temperatures. Optimum temperature and pH for GEH activity were 45°C and 9.0, respectively. The enzyme was either inhibited or stimulated by calcium chloride and sodium desoxycholate depending on the concentration of the salt. Sodium taurocholate was inhibitory. The enzyme displayed highest activity toward tributyrin followed by butter oil, olive oil, soybean oil and corn oil.

To my wife, Lila, and
my son, Almir. Yes,
we are together now.

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INTRODUCTION

Ripening of fermented dairy products is a complex mechanism. Metabolic transformation of fat and proteins by microorganisms, intentionally added or as contaminants give formation to a number of products. These components when present in a well balanced proportion are believed to be responsible for the characteristic flavor of a fermented product. A shift in the balance in the components leads to stronger or weaker flavor or even an undesirable flavor. Added enzymes such as rennet, proteases and lipase powder of glandular origin (kid, calf and lamb) and milk enzymes have been considered as possible sources of typical and atypical flavor formation.

Research in cheese ripening has established that starter microorganisms play an important role in cheese flavor formation. Experiments performed under aseptic conditions where only lactic acid bacteria were present during cheese making have shown that lactic streptococci are able to produce the characteristic Cheddar cheese flavor. In yogurt, fat metabolism may be involved in flavor development. It is not firmly established whether the volatile fatty acids present in yogurt are derived from fat or protein metabolism by yogurt starter enzymes. Also, free fatty

acid formation may be attributed in part to the enzymatic action of thermoresistant lipases of contaminating psychrotrophic bacteria.

Lipolysis by lactic acid bacteria in dairy products appears to be a slow process which increases as the microbial population decreases. It is generally accepted that the microorganisms release their enzymes to the surrounding media upon their death. The microbial species as well as strains of a particular microorganism appear to affect the nature and activity of the released enzyme.

Streptococcus thermophilus is commonly utilized as a starter organism in a number of cheeses and yogurt. Presently little or no information is available relative to the factors influencing lipase production, activity, specificity and stability of the enzyme. Accordingly a study was undertaken to screen various strains of Streptococcus thermophilus for glycerol ester hydrolase activity. Also certain factors affecting lipase production and lipase activity were studied to clarify the role of the microbial lipase in fermented dairy products.

LITERATURE REVIEW

Concept of Lipase

Based on the specific "physiological" task it performs, Desnuelle (1961) gave a very strict concept of lipase. Thus, pancreatic lipase was defined as an enzyme capable of hydrolyzing an emulsion of long chain triglycerides in the duodenum.

Lawrence (1967) described lipolytic enzymes as substances capable of catalysing the hydrolysis of ester linkages in lipids with the release of the constituent alcohol and acid moieties.

According to Brockerhoff and Jensen (1974) lipolytic enzymes may be defined as long chain fatty acid ester hydrolases. Long chain fatty acids include aliphatic acids, saturated or unsaturated with 12 or more carbon atoms. They emphasize that any esterase capable of hydrolyzing esters of oleic acid is a lipolytic enzyme.

Shahani (1975) suggested that lipase may be defined as an enzyme which hydrolyzes the esters at an oil-water interface in an insoluble or heterogeneous system.

Richardson (1976) defined lipases as enzymes that hydrolyze ester linkages of emulsified glycerides at an oil-water interface.

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The concept of lipase by Brockerhoff and Jensen (1974) is more restrictive since it does not include short chain fatty acid esters as a substrate for lipases (e.g. C_4, C_6, C_8, C_{10}). Nonetheless, it includes esterases in lipase category.

Lipase concept by Lawrence (1967) includes attack on any ester linkage in lipids. Esterases are not to be considered lipases, since the soluble substrate on which they act is not usually considered in this class of lipids. This point of view is in clear agreement with those of Shahani (1975) and Richardson (1976) which limit the substrate to an oil-water interface. Also, according to Shahani (1978) the major difference between the lipases and esterases seems to be associated with the state of the substrate they act upon. While esterases can hydrolyze soluble or fully dissolved substrates, the lipases cannot.

Nomenclature

Lipolytic enzymes are hydrolases and belong to Enzyme Class 3 according to the classification recommended by the Enzyme Commission of the International Union of Biochemistry (1961). All lipolytic enzymes are ester hydrolases, Enzyme Group 3.1, with the single exception of the ceramidase, a C-N hydrolyzing enzyme (E.C.3.5.). Since in the narrowest sense they hydrolyze esters of fatty acids, they are carboxyl ester hydrolases (E.C.3.1.1.) (Brockerhoff and

Jensen, 1974). The International Union of Biochemistry defined lipase as glycerol ester hydrolases (E.C.3.1.1.3.).

Triglyceride Hydrolysis by Lipase

In general, lipases hydrolyze triglycerides in a stepwise fashion as follows:

$$\text{Triglyceride} \rightarrow \text{1,2 Diglyceride} + \text{Free Fatty Acid} \rightarrow \text{2 Monoglyceride} + \text{2,3 Diglyceride} + \text{Free Fatty Acid}$$

However, depending on both positional and fatty acid specificities of the enzyme some exceptions occur. The reaction shown is for lipases with primary ester specificity which seems to be the predominant type (Richardson, 1976).

Specificities of Lipases

According to Desnuelle and Savary (1963) by substrate specificity is understood the influence exerted on lipase activity by the physical state and chemical nature of the substrate. Other specificities are: (1) positional specificity which can be defined as the ability to hydrolyze only the primary or both primary and secondary ester bonds of a triglyceride. In the latter case more than one enzyme is present; (2) stereospecificity is the ability to hydrolyze only ester 1 or only ester 3 of the triglyceride; (3) glyceride specificity is characterized by a preferential

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hydrolysis of low molecular weight triglycerides over high molecular weight substrate; (4) Fatty acid specificity occurs when one type of fatty acid is more rapidly hydrolyzed than another type, both being attached to the same positions of like triglycerides molecules (Richardson, 1976; Brockerhoff and Jensen, 1974).

Among the microorganisms, the purified extracellular lipase of Geotrichum candidum has been exhaustively studied. The lipase is characterized by a unique, but not absolute specificity for fatty acids containing Cis-9 or Cis, Cis 9, 12 unsaturation, hydrolyzing both, regardless of position within the triglyceride molecule (Jensen, 1974).

Lactic acid bacteria reportedly show systematic preference for short chain fatty acids, mainly C_3 and C_4 (Fryer et al., 1967; Umemoto et al., 1968; Oterholm et al., 1970; Dovat et al., 1970; Chander et al., 1973; Formisano et al., 1974; Chander et al., 1979). These bacteria have been shown to hydrolyze more complex lipids containing higher fatty acids and natural lipids (Umemoto et al., 1968; Angeles and Marth, 1971; Chander et al., 1979).

Kinetics of Lipolysis

Brockerhoff and Jensen (1974) stated that although the hydrolysis of triglycerides by pancreatic lipase, as demonstrated by Sarda and Desnuelle (1958) obeys the Michaelis-Menten equation:

$$v_o = \frac{Vmax\ (S)}{km\ (S)},$$

the appropriate kinetics for a lipolytic enzyme have yet to be developed. They argue that conventional enzyme kinetics have been developed for reaction in aqueous solution.

The enzymatic hydrolysis of a lipid is different in that the enzyme is water-soluble but the substrate is not. Therefore, the enzyme-substrate interaction must take place at the interface of the aggregate substrate and water. Accordingly, the kinetic treatment of such enzymatic reaction is still in the embryonic stage. It should also be mentioned that since lipase action takes place at an oil/water interface, this rather unique situation gives rise to variables not ordinarily encountered in enzyme reactions. Factors such as the amount of surface area available, the permeability of the emulsion, the type of glyceride employed, the physical state of the substrate (complete solid, complete liquid, or solid-liquid) and the degree of agitation of the reaction medium, must be taken into account for the results to be meaningful.

Concept of Lipolysis in Milk

According to Downey (1975) lipolysis results from the enzyme splitting of the milk fat leading to the accumulation of free fatty acids many of which possess rather pungent

flavors and contribute, for example, to rancid flavor defects in foods.

Milk fat in normal milk is surrounded by a membrane and is therefore largely protected from attack by the lipolytic enzymes in milk. However, if the milk is subjected to excess agitation or turbulence, the fat globule membrane may be disrupted and a new membrane is formed by casein and associated lipolytic enzymes.

Additionally several factors can contribute to increase or reduce the rate of lipolysis in normal milk. Thus, green pasture feeding decreases and dry feed increases the incidence of rancidity (Chen and Bates, 1962). A poor quality ration with a low energy level can increase the incidence of rancidity (Gholson et al., 1966) as can a high carbohydrate diet (Kodgeve and Rachev, 1970). Advanced lactation (Colmey et al., 1957), Mastitis (Guthrie and Herrington, 1960), Estrous (Wells et al., 1969) and pipeline milkers (Thomas et al., 1955) also tend to accelerate milk fat lipolysis.

Lipolysis in Cheese and Flavor Formation

Harper (1959) related cheese flavor to the proper balance of components which can contribute to both taste and odor. Thus, the proportion of both amino acids and fatty acids are important in relation to cheese flavor. In Provolone cheese, a 2:1 glutamic acid:butyric acid ratio was desirable while a definite, characteristic flavor was

obtained in Swiss cheese when propionic acid and proline were present in a ratio of 2:1.

Fryer (1969) listed a series of components which would be responsible not only for cheese flavor but also for a range of food flavors. The more important components included certain fatty acids, methyl ketones, aldehydes, diacetyl, amines, peptides, and sulfur compounds. Other components such as amino acids, esters, alcohols, partial glycerides and salts probably would have some effect on the typical cheese flavor.

Kosikowski and Mocquot (1958) created the Component Balance Theory that recognizes that a slight alteration in the proportion of the basic components results in a slight change in the Cheddar flavor, while the disproportional increase of certain components can lead to off-flavor formation. An intensification of the cheese flavor is possible when all the components increase proportionally. Consequently, even when in their individual state, these components have flavor other than cheese flavor, collectively when in a critical balance, the resulting cheese flavor is typical (Kosikowski, 1978).

Role of Microbial Lipases in Cheese Ripening

The primary interest in lipolytic microorganisms has been related to spoilage of dairy products containing relatively high concentrations of fat. Most natural fats are

composed of glycerides of long chain fatty acids, saturated as well as unsaturated, which are subjected to enzymatic hydrolysis (Dugan, 1976).

According to Kurtz (1974), milk fat consists chiefly of triglycerides of fatty acids (95-96%). It also contains varying quantities of other compounds as shown in Table 1.

Lipolysis of milk fat has been intensively studied since a number of milk products and milk fat containing products are commercially and nutritionally important.

Although off-flavor production by microorganisms is undesirable, there are instances in which fat hydrolysis contributes to improvement of the flavor of milk products. Fatty acid production as it occurs in Blue, Cheddar, Gouda, Romano, and Swiss cheeses is essential to development of the characteristic flavor of these products (Mabbit and Zielinska, 1956; Oterholm et al., 1970a; Alford et al., 1971; Angeles and Marth, 1971).

Reiter and Sharpe (1971) reported that free fatty acids are not the components per se of flavor. However, they pointed out that carbonyl compounds might be derived by the oxidation of oleic, linoleic and arachidonic acids.

Acceleration of flavor production in Cheddar cheese by addition of enzymes has been reported. Richardson et al. (1971) reported that Cheddar and Provolone cheeses were organoleptically preferred when gastric lipase preparations were included in their manufacture.

Table 1. Composition of bovine milk lipids[a]

Class of lipid	% Total milk lipids
Triglycerides of fatty acids	95-96
Diglycerides	1.26-1.59
Monoglycerides	0.016-0.038
Keto acid glycerides (total)	0.85-1.28
Ketonogenic glycerides	0.03-0.13
Hydroxy acid glycerides (total)	0.60-0.78
Lactonogenic glycerides	0.06
Neutral glyceryl ethers	0.016-0.020
Neutral plasmalogens	0.04
Free fatty acids	0.10-0.44
Phospholipids (total)	0.80-1.00
Sphingolipids (less sphingomyelin)	0.06
Sterols	0.22-0.41
Squalene	0.007
Carotenoids	0.0007-0.0009
Vitamin A[b]	0.0006-0.0009
Vitamin D	0.00000085-0.0000021
Vitamin E	0.0024
Vitamin K	0.0001

[a]Kurtz (1974)

[b]Based on the free alcohol

Kosikowski and Iwasaki (1974) studied the contribution of commercial enzyme preparations to Cheddar cheese flavor development. They found that at higher enzyme levels rancidity was noticeable but at lower amounts it was muted and the cheese taste was rather pleasant.

It has been known that bacteria can produce fatty acids from a variety of substrates. Thus, Platt and Foster (1958) reported that glucose metabolism by homofermentative lactic streptococci, under anaerobic conditions, gave formation to acetic and formic acids.

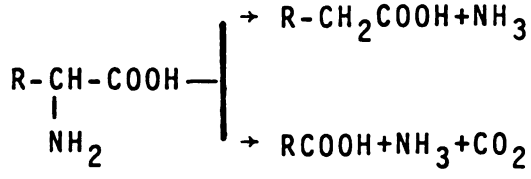
Nakae and Elliot (1965) showed lactic acid bacteria (Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis, and lactobacilli) produced free fatty acids from casein hydrolyzate. Total free fatty acids produced by lactobacilli were higher than those by lactic streptococci. The highest value of total free fatty acid in lactic streptococci was obtained from Streptococcus diacetilactis. Acetic acid was the main acid produced by all the strains and it varied between 79% of the total free fatty acid produced by Streptococcus lactis and 95% by one lactobacilli strain. Other acids produced were: C_3, C_4, C_5 and C_6.

Nakae and Elliot (1965a) also reported that one strain of Streptococcus diacetilactis and one of Lactobacillus were capable of producing acetate from alanine, glycine and serine; propionate from threonine; isobutyrate from valine and isovalerate or valerate from leucine and isoleucine.

They pointed out that these reactions may be ascribed mainly to oxidative deamination and decarboxylation.

Kowalewska et al. (1971) showed that lactic acid bacteria produced free fatty acids from casein substrate at pH values 5.5 and 6.5. All strains of lactic acid bacteria (Streptococcus lactis, Streptococcus diacetilactis, Streptococcus cremoris) specially Streptococcus cremoris produced butyric acid.

Nakae et al. (1974) found that an increase of volatile fatty acids was preceded by that of free tyrosine in skim milk. They proposed a mechanism of volatile acid formation from amino acids as shown:



Milk fat appears to be the main source of free fatty acids in milk products. Several reports have been published relative to fat hydrolysis by lactic acid bacteria and other microorganisms.

Peterson and Johnson (1949) tested 54 lactobacilli and 8 micrococci isolated from cheese. The cultures were incubated in a butterfat substrate, pH 5.0-6.0, at 30°C for 60 days. They found that 12 lactobacilli and 4 micrococci displayed positive lipolytic effects. All lipolytic

lactobacilli were identified as Lactobacillus casei while the micrococci were identified as Micrococcus freudereichii, Micrococcus caseolyticus and Micrococcus conglomeratus.

Stadhouders and Mulder (1958) studied the effect of lactic acid bacteria on the hydrolysis of fat in cream. Their results agreed with those of Peterson and Johnson (1949). However, they questioned whether the bacteria are able to hydrolyze fat in cheese as well as in cream. They argued that in cream the bacterial count may be of the order of several billion/ml but in cheese the count of lactobacilli generally does not exceed 40-50 million/g. They concluded that lactic acid bacteria were of no importance for fat hydrolysis in cheese. In a subsequent study, Stadhouders and Mulder (1960) reported the influence of several sources of lipases in cheese aseptically manufactured from aseptically drawn milk. They concluded that milk lipase was of little importance in the hydrolysis of fat in cheese from pasteurized milk. Further, the thermoresistant lipases from representatives of the genera Pseudomonas and Achromobacter increased the hydrolysis of the fat in the cheese. Upon increasing the pasteurization temperature, the lipases of these gram negative rods were destroyed progressively.

Reiter et al. (1967) showed that cheese made under aseptic conditions with the addition of starter displayed weak but consistent milk fat hydrolysis and typical Cheddar flavor. Streptococcus diacetilactis was considered to be of

major importance in the production of free fatty acids. A control cheese made with gluconic acid lactone was devoid of fat hydrolysis as well as of cheese flavor.

Sovietskii and Kostroma cheeses were manufactured with several strains of lactic bacteria of different degree of G.E.H. activity (Umanskii et al., 1976; Belov et al., 1974; Umanskii and Borovkova, 1980). Cheeses made by using starters of higher G.E.H. activities received a higher organoleptic score, when compared with cheese made with starters of lower G.E.H. activities. Among the lactic streptococci, Streptococcus diacetilactis displayed highest lipolytic effects.

Umemoto and Sato (1975) investigated the relationship between fat hydrolysis and lipolytic activity of lactic acid bacteria participating in Cheddar cheese ripening. At the age of 150 days, 70% of the bacteria isolated from cheese were identified as Lactobacillus casei and Lactobacillus plantarum. They supported the view that lactobacilli and streptococci participate in fat hydrolysis during cheese ripening. However, they did not take into consideration the role of thermoresistant microbial lipases likely to be present in the cheese milk.

The difference in results observed between Stadhoulders and Mulder (1960) and other cited workers could be explained by three factors:

a) The strain of lactic acid bacteria employed since they present different degree of G.E.H. activity (Searles et al., 1970; Dovat et al., 1970).

b) The method employed to detect the free fatty acids.

c) The degree of integrity of the triglycerides, since G.E.H. of lactic acid bacteria is more active toward mono and diglycerides than toward triglycerides (Stahdouders and Veringa, 1973).

Contrary to the earlier findings, Stadhouders and Veringa (1973) reported that the hydrolysis of the triglycerides of milk fat by lactic bacteria, although limited, may still be significant in cheese.

Angeles and Marth (1971) studied the lipolytic activity of lactic bacteria in soybean oil and soy milk. They found that Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetilactis, Streptococcus thermophilus, Leuconostoc mesenteroides, Pediococcus cerevisiae, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus pentosus and Lactobacillus brevis were able to hydrolyze tributyrin and triolein, but not soybean oil. However, free fatty acids were detected in the medium when soy milk was used as substrate. The authors inferred that these fatty acids were resulting from the lipolytic activity of Lactobacillus casei, Lactobacillus delbrueckii, and Streptococcus thermophilus. Another possible explanation is the metabolism of amino acids with production of free fatty acids as previously

discussed.

Factors Affecting Microbial Lipase Production

Lipase appears to be a universal enzyme among microorganisms (Fryer et al., 1967). The amount produced varies depending on the specie and even between two strains of the same microorganism. The environment plays an important role in lipase production and a number of variables can contribute either to increased or decreased lipase production.

Effect of Incubation Temperature

Literature reports show in general 21-22°C as the most stimulating temperature for microbial lipase production (Lawrence et al., 1967; Jonsson and Snigg, 1974; Nashif and Nelson, 1953c). Specifically, the following optimum temperatures for lipase production have been reported: Mycotorula lipolytica, 30°F (Peters and Nelson, 1948): Pseudomonas fragi, 15°C or below (Nashif and Nelson, 1953a); Achromobacter lipolyticum, 32°C; Pseudomonas aeruginosa, 21°C; Flavobacterium sp., 21°C; Pseudomonas synxantha, 21°C; Pseudomonas viscosa, 21°C; Alcaligenes viscosus, 21°C; Pseudomonas sp., 21°C; Serratia marcescens, 21°C; Pseudomonas fluorescens, 15°C (Nashif and Nelson, 1953c); Pseudomonas fluorescens, 20°C (Alford and Elliot, 1960); Achromobacter lipolyticum, 21°C (Khan et al., 1967); Pseudomonas fragi, 22°C (Lawrence et al., 1967); Staphylococcus aureus, 37°C (Mates and Sudakevitz, 1973); Bacillus licheniformis, 20°C; Micrococcus caseolyticus, 20°C; Saccharomycopsis lipolytica, 20°C

(Jonsson and Snigg, 1974); Aspergillus wentii, 30°C (Chander et al., 1980). Consequently the optimum temperature for lipase production has to be established for each microorganism individually. Also, lipase production must be determined at intervals of time during incubation, since it is known that after a determined period of incubation, lipase production reaches a peak, followed by a decrease (Lawrence et al., 1967).

Disagreement between the results of Nashif and Nelson (1953a) and Lawrence et al. (1967) for the best temperature for lipase production by Pseudomonas fragi (15° and 22°C) respectively, might have occurred because the latter workers did not grow the microorganism at 15°C.

In relation to Streptococcus thermophilus there appears to be no report on the influence of temperature on lipase production.

Effect of pH of Growth Medium on Lipase Production

The initial pH of the growth medium is important for lipase production (Lawrence, 1967). Most of the studies relative to the influence of pH on lipase production were conducted without constant pH. The optimum pH for lipase production appears to be dependent on the specie, and strains of the same microorganism may exhibit widely different behavior. Vadehra and Harmon (1969) determined the best range for lipase production by Staphylococcus aureus to be between 7.5-9.0. Mates and Sudakevitz (1973) and Jonsson

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and Snigg (1974) reported the best pH to be 7.5 and 8.0, respectively. Mates and Sudakevitz (1973) did not find lipase production by the microorganism at pH 8.5 and 9.0 after 48 h of incubation.

Chander et al. (1977, 1980) found pH 6.0 to be the optimum for lipase production by Penicillium chrysogenum and Aspergillus wentii.

Niki et al. (1966) reported pH 8.0 as the optimum for extracellular lipase production by Penicillium roqueforti in whey. Acidified whey was apparently unsuitable. It may be significant that protease formation was high at pH 4.0 or below and practically no extracellular protease formation was observed at neutral and alkaline whey. This was completely opposed to the results obtained on protease formation. In general, pH 5.0 and 8.5 appears to reduce considerably lipase production by microorganisms. However an exception was reported by Peters and Nelson (1948) that found pH 4.5 as the best for lipase production by Mycotorula lipolytica.

Nashif and Nelson (1953b) reported that Pseudomonas fragi was not able to produce lipase below pH 4.5. They supported the view that at lower pH prevailing in fermented dairy products no lipase is produced.

A search of the literature revealed no reports relative to the effect of the pH medium on lipase production by Streptococcus thermophilus.

Most microorganisms are capable of modifying the pH of culture medium during growth. Many workers only report the initial pH of the culture medium without registering the pH changes occurring during incubation. To overcome this problem, the optimum pH for lipase production by microorganisms should be determined under pH stat conditions.

Effect of Adding Lipids to the Growth Medium on Lipase Production

The adaptative character of lipase of certain microorganisms was demonstrated by Cutchins et al. (1952). The latter workers stated that bacterial lipase production was stimulated by the presence of the specific substrate or else that the elaboration of the enzyme depended upon the preferential utilization of certain substrates by the organism. Khan et al. (1967) found that addition of 1% milk fat, corn oil, or olive oil to the growth medium increased lipase production of Achromobacter lipolyticum by 25.0, 68.8, and 87.5%, respectively, when compared with a control containing no oil.

Iwai et al. (1973) suggested that Geotrichum candidum inducibly produced lipase in the presence of higher fatty acids or oils in the medium. Oleic and linoleic acids played a significant role as the inducer for lipase production. On the other hand, Chander et al. (1979) reported inhibitory effect of addition of oleic acid to the growth medium on lipase production by Streptococcus faecalis.

However, C_3, C_4, C_6 and C_8 were stimulatory and long chain saturated fatty acids (C_{12}, C_{14}, C_{16} and C_{18}) slightly decreased lipase production.

Many workers have found addition of lipids to the growth medium to be inhibitory to lipase production by microorganisms (Eitenmiller et al., 1970; Mates and Sudakevitz, 1973; Chander et al., 1980, 1981). This effect has been attributed to the bacteriostatic effect of unsaturated fatty acids (Eitenmiller et al., 1970) or, according to Maxcy and Chandan (1962) to a physical phenomenon of surface tension lowering by the fatty acids causing interference with the metabolism of the microorganism.

Effect of Nitrogenous Nutrients on Lipase Production

Most of the foods undergoing lipolysis also contain proteins and other nitrogenous materials as a major component. Various culture media utilized in lipase production studies contain proteins, or more frequently, protein degradation products. Cutchins et al. (1952) suggested that lipase production decreased as the structural complexity of the nitrogenous source increased. A casein hydrolyzate inhibited completely the lipolytic activity of Pseudomonas fluorescens, while glycine stimulated lipase production more than a peptone did.

Nashif and Nelson (1953a) investigating the effect of different protein digests and hydrolyzates on lipase production by Pseudomonas fragi, found great variance among the

different commercial preparations, indicating that the character of the nitrogen source is an important factor. Lawrence et al. (1967) confirmed the variation in results found by Nashif and Nelson (1953a), and furthermore established that Bacto-Peptone (DIFCO) was the most satisfactory nitrogen source for lipase production by Pseudomonas fragi and Micrococcus freundereichii.

Searles et al. (1970) found no significant change in the lipolytic activity of 12 lactic cultures when 0.2% casein was added to the growth medium.

Chander and Ranganathan (1975) found that alanine, glycine, lysine, and serine appeared to be stimulatory to lipase production by Streptococcus faecalis. Aspartic acid, cystine, phenylalanine, proline and tyrosine, on the other hand, appeared not to influence lipase production.

Beside the nature of the nitrogen source, the microorganisms also differ in their response to different types of nitrogen sources. Thus, while Bacto peptone stimulated production of lipase by Pseudomonas (Lawrence et al., 1967), the same product inhibited lipase production by Staphylococcus aureus, although cellular growth was normal (Mates and Sudakevitz, 1973).

Factors Affecting Microbial Lipase Activity

Temperature

Optimum temperature for lipase activity appears to be limited to the range of 30^{o}-40^{o}C except for a few reported

exceptions. A purified Torulopsis sp. lipase was found to have an optimum at 45°C (Lawrence, 1967). The crystalline lipase from Aspergillus niger was reported to have an optimum at 25°C (Lawrence, 1967). Oterholm et al. (1970) found 47°C to be the best temperature for lipase activity of a purified lipase of Propionibacterium shermanii.

Umemoto et al. (1968) reported that the optimum temperature for lipolytic activity of cell-free extracts of Lactobacillus plantarum and Lactobacillus casei to be 37°C when tributyrin and butterfat were used as substrates. They also reported 45°C as the optimum temperature for lipase activity of Streptococcus diacetilactis toward tributyrin. Khan et al. (1967) found 37°C to be the optimum temperature for lipase activity of the extracellular lipase of Achromobacter lipolyticum. The same temperature was reported by Hugo and Beveridge (1962) for a strain of Serratia marcescens and by Eitenmiller et al. (1970) for Penicillium roqueforti lipase. Tsujisaka et al. (1973) reported optimum temperature for lipolytic activity of a strain of Geotrichum candidum in olive oil to be 40°C. Similar results were reported for a strain of Streptococcus faecalis toward butter fat substrate (Chander et al., 1979a).

Although most of the microbial lipases present their best temperature for activity between 30-40°C, they are also capable of hydrolyzing lipids at very low temperatures. It was demonstrated by Alford and Pierce (1961) that lipase

activity of Pseudomonas fragi, Geotrichum candidum, Penicillium roqueforti, Staphylococcus aureus, Candida lipolytica and Penicillium sp. was discernible after one week of incubation at -18°C.

pH Optimum

The effect of pH on the rate of hydrolysis by lipase is influenced by the stability of the enzyme, the velocity of the enzyme-substrate combination and breakdown, and the properties of the substrate-aqueous phase interface (Lawrence, 1967).

The optimum pH varies for the same microorganism, depending on the buffer, temperature and other conditions. Hugo and Beveridge (1962) showed that the optimum pH for lipolytic activity of Bacillus cereus was 7.2, 7.9, 7.2, and 10.4 respectively, depending on whether tributyrin, ethyl laurate, olive oil or ethyl butyrate were used as substrate. Several pH optima reported by different workers were: Penicillium roqueforti, 6.5-6.8 (Fodor and Chari, 1949); Penicillium roqueforti, 5.5 (Shipe, 1951); Penicillium roqueforti, 8.0 (Eitenmiller et al., 1970); Achromobacter lipolyticum, 9.0 (Intracellular lipase) (Shahani et al., 1964); Achromobacter lipolyticum, 7.0 (extracellular lipase) (Khan et al., 1967); Micrococcus lipolyticus, 5.0 (Alford and Pierce, 1963); Mucor javanicus, 7.0 (Saiki et al., 1969); Pseudomonas aeruginosa, 8.9 (Finkelstein et al., 1970); Saccharomycopsis lipolytica, 9.5; Micrococcus caseolyticus, 9.5; Bacillus

licheniformis, 8.5; Staphylococcus sp., 8.5 (Jonsson and Snigg, 1974).

Among the lactic bacteria a few reports showed the following values for optimum pH for lipolytic activity: Lactobacillus casei, 7.0; Lactobacillus plantarum, 7.0-8.0; Lactobacillus helveticus, 7.0; Streptococcus diacetilactis, 6.0-7.0 (Umemoto et al., 1968); Lactobacillus brevis, 6.5 (Chander et al., 1973).

Variations in the optimum pH for Penicillium roqueforti lipase may be due to the use of different substrates, different assay conditions and different strains.

Effect of Addition of Bile Salts and Calcium Chloride to the Assay Emulsion

Enhanced microbial G.E.H. activity by addition of bile salts to the assay substrate has been reported (Shahani et al., 1964; Oi et al., 1969; Nagaoka and Yamada, 1973; Yamaguchi et al., 1973; Sugiura et al., 1974).

However, an inhibitory effect also has been observed (Saiki et al., 1969; Finkelstein et al., 1970; Oterholm et al., 1970).

The nature of the effect whether inhibitory or stimulatory depends on variables such as the degree of enzyme purification, the bile salt utilized, the salt concentration, the enzyme producing microorganism and the enzyme itself.

Shipe (1951) studying lipase of Penicillium roqueforti and Aspergillus niger found that addition of 10 mg of

$CaCl_2$/12 ml of substrate almost doubled the activity of both lipases. However, as the concentration of $CaCl_2$ was increased to 20 mg, the enzyme activity began to decrease. Calcium chloride activation of G.E.H. of Aspergillus niger was confirmed by Iwai et al. (1964). Shah and Wilson (1963) suggested that the egg yolk factor in Staphylococcus aureus was a lipase with a specific requirement for a fatty acid acceptor such as calcium ions.

Rhizopus lipase was stimulated by the addition of calcium chloride and ferric sulfate to the substrate (Oi et al., 1969).

Although calcium chloride was stimulatory to the lipase activity of Achromobacter lipolyticum, several other salts were more stimulatory, including magnesium chloride, sodium sulfate, magnesium sulfate, and sodium chloride (Khan et al., 1967).

Calcium chloride was found not to influence lipase activity of Mycoplasm (Rotten and Razin, 1964), and conversely to the finding of Shipe (1951), Eitenmiller et al. (1970) did not observe any appreciable stimulatory effect of calcium chloride on the activity of Penicillium roqueforti lipase.

Calcium chloride was also reported to be inhibitory to lipase of Mucor javanicus (Saiki et al., 1968) and to a G.E.H. of Propionibacterium shermanii (Oterholm et al., 1970).

According to Wills (1965) bile salts probably exert their action by increasing the interfacial area of the

triglyceride-water phase.

The utilization of calcium chloride as a stimulatory agent for lipase activity is based on two hypothesis: (1) Calcium ions inhibit the resynthesis of ester linkage, which would effectively shift the reaction in the direction of hydrolysis (Lawrence, 1967); (2) Calcium soaps activate the hydrolysis by changing the interfacial substrate-water relationship to favor enzyme action (Iwai et al., 1964).

Nature of the Microbial Lipolytic Enzyme

According to Stanier et al. (1976), three distinct types of microbial enzymes are: (1) exoenzymes synthesized within the cell and excreted into the medium; (2) periplasmic enzymes, present in the space between the cell membrane and the outer layer of the wall; (3) exocellular enzymes, which act upon substrates which are external to the cell membranes but remain tightly bound to the cell surface.

Pollock (1962) classified the enzymes as cell bound which are carried along with the cells upon centrifugation or extracellular.

According to Lawrence (1967), extracellular lipase production should be followed in young cultures, preferably during the logarithmic phase of growth when they are less prone to cell lysis. It is justified, since in some instances the occurrence of enzymes in the culture fluids is the result of cell lysis.

Several studies have been reported on the nature of lypolytic enzymes of lactic acid bacteria. A consensus of the reports indicates that lactic acid bacteria have only intracellular or cell-bound enzymes (Peterson and Johnson, 1949; Searles et al., 1967; Umemoto et al., 1968; Oterholm et al., 1968; Morichi et al., 1968; Chandan et al., 1969; Chander and Chebbi, 1972; Formisano et al., 1974).

However, Fryer et al. (1967) studying the lipolytic activity of 56 strains of lactic bacteria (Streptococcus lactis, Streptococcus diacetilactis, Streptococcus cremoris, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus brevis, Pediococcus cerevisiae, and Lactobacillus mesenteroides) reported no lag in lipolysis under the assay conditions. Accordingly they concluded that the lipase was close to the cell surface, was an exoenzyme and not an intracellular. It appears that enzyme location classification rather than the lipase position in the cell envelop is responsible for the different conclusions between the works of Reiter et al. (1967) and other cited workers. Thus, the term intracellular enzyme should only be used for enzymes present only in the cytoplasm portion of the cell, while the membrane related enzymes should be classified as exocellular or cell bound enzymes.

Thermostability of Microbial Lipases

Thermostability of microbial lipases has been frequently reported linked to their influence in spoilage and flavor

formation in milk and milk products (Nashif and Nelson, 1953b; Stadhouders and Mulder, 1960; Pinheiro et al., 1965; Law, 1979).

Despite the advantages of storing milk at low temperature, psychrotrophic bacteria can multiply below 4°C irrespective of their optimum growth temperature. Although most of the psychrotrophic microorganisms are heat labile at pasteurization temperature, they produce thermostable lipases which are released to the medium upon autolysis.

Nashif and Nelson (1953b) reported that over 50% of the lipase produced by Pseudomonas fragi was not inactivated by pasteurization of cream at 71.5°C for 30 min. Accordingly, they observed that butter containing residual lipase underwent considerable fat degradation even during storage at -10°C. The fat breakdown increased by storage at 5°C or higher temperature.

Stadhouders and Mulder (1960) found lipase from Pseudomonas and the Achromobacteriaceae to be rather thermoresistant. They added cultures of these microorganisms to milk before pasteurization and fat hydrolysis in the cheese prepared from the pasteurized milk was increased.

Pinheiro et al. (1965) also reported thermoresistant lipolytic enzymes from Pseudomonas microorganisms. The enzymes caused off-flavors in Purdue Swiss-type cheese enough to make it unmarketable.

A report by Chander et al. (1979) showed that inactivation of a purified lipase of Streptococcus faecalis was 60% by heating at 60°C/10 min. Complete inactivation was reported by heating the enzyme to 90°C for 10 min.

Characteristics of Streptococcus thermophilus

According to the Bergey's Manual of Determinative Bacteriology (1974) Streptococcus thermophilus is Gram positive, catalase negative, facultatively anaerobic organism with spherical or ovoid cell, 0.7 to 0.9 μm in diameter, occurring in pairs to long chains. Optimum growth temperature is between 40 and 45°C. Growth occurs at 50°C but not at 53°C. No growth is observed below 20°C. The microorganism survives a heat treatment of 65°C for 30 min. The specie is easily recognized by its high growth temperature, thermal tolerance, inability either to ferment maltose or to grow in media containing 2% or more sodium chloride.

Acid is produced from glucose, fructose, lactose and sucrose. No acid is observed from trehalose, inulin, glycerol, mannitol, sorbitol and salicin, and rarely from raffinose, xylose or arabinose. Common sources of the microorganism are: milk and milk products such as Swiss cheese and yogurt.

Biochemical Characteristics

Rasic and Kurman (1978) gave the following description of the microorganism: Streptococcus thermophilus belongs to

the homofermentative group of lactic acid bacteria and produces lactic acid from sugar in yields ranging from 85% to 98% including small quantities of other products. In milk it produces 0.7-0.8% L (+) lactic acid; some strains are capable of producing up to 1% lactic acid.

The proportion and composition of by products depend on culturing conditions and strains. In a glucose medium with no pH control, Streptococcus thermophilus produces anaerobically formic and acetic acids, CO_2, ethanol and acetoin, in addition to lactic acid. The products alter in character at pH 7.0 (Platt and Foster, 1958). In milk, it produces volatile acids such as formic, acetic propionic, butyric, isovaleric and caproic (Veringa et al., 1968; Turcic et al., 1969), acetoin, small amounts of acetaldehyde and acetone (Goerner et al., 1968; Bottazzi and Vescovo, 1969), ethanol and butanone 2 (Goerner et al., 1972). Some strains also produce diacetyl (Davis, 1956; Rasic and Milanovic, 1966).

Streptococcus thermophilus shows very weak proteolytic activity in milk, and most of the liberated amino acids are consumed during the lagarithmic phase of growth.

Some strains of this microorganism are antagonistic to a number of microorganisms. Pulusani et al. (1979) demonstrated antimicrobial activity of a methanol-acetone extract from Streptococcus thermophilus against Pseudomonas aeruginosa, Pseudomonas fluorescens, Bacillus subtilis,

Bacillus sp., Pseudomonas sp., Flavobacterium, Salmonella typhimurium, Shigella sp., Escherichia coli and various strains of Streptococcus lactis. The authors inferred that the antimicrobial compounds were heat stable amines of low molecular weight.

EXPERIMENTAL PROCEDURES

Culture Source

Thirty two strains of Streptococcus thermophilus were obtained through the courtesy of Dr. G.A. Somkuti, Eastern Regional Research Center, United States Department of Agriculture, Philadelphia, PA. Identification tests at the center showed that except for strains ST/AH and Is which showed growth in the presence of 2% NaCl, all the strains were considered typical Streptococcus thermophilus. Table 2 shows the cultures and their origin.

Culture Maintenance

The cultures were maintained in both sterile 10% reconstituted non-fat dry milk and in Hogg and Jago (1970) medium to which 1% lactose was added (Appendix page 98). The cultures were incubated at 37°C for 12-18 h and stored at 4°C. All the cultures were transferred weekly to fresh media.

Cell Growth and Harvesting

To screen for G.E.H. activity, the 32 strains were grown in Hogg and Jago medium containing 1% lactose. Two-liter Erlenmeyer flasks containing 1.4 l of the sterilized medium were inoculated with 0.5% of a 18 h culture and incubated

Table 2. *Streptococcus thermophilus* strains used in the studies

Strain	Origin[a]	Strain	Origin
4074	Hansen	EB-8	UN
4109	"	MC	"
6031	"	14485	ATCC
6069	"	19258	"
6097	"	19987	"
7024	"	3641	NRRL
7132	"	371	NIZO
9353	"	L225	"
ST-4	Miles	Ds	"
ST-3	Microlife	Is	"
ST-7	"	Sts	"
ST-8	"	SFi-1	Nestle
ST-9	"	SFi-3	"
MLT-33	"	CNRZ 391	INRA
ST/AH	"	CNRZ 404	"
YB/ST	"	CNRZ 406	"

[a]Hansen, Chr. Hansen's Laboratory, Inc.
Microlife, Microlife Technics
Miles, Miles Laboratories, Inc.
ATCC, American Type Culture Collection
NIZO, Netherland Institute for Dairy Research, Ede, The Netherlands
UN, University of Nebraska
INRA, National Agricultural Research Institute, Jouy-Eu-Josas, France
NRRL, Northern Regional Research Center, U.S. Department of Agriculture
Nestle, Nestle Products Ltd., Lausanne, Switzerland.

in stationary state at 37°C for 18 h. After growth, the cells were harvested by centrifugation at 16,600xg for 10 min in a Sorvall SS-1 Super Speed Angle Centrifuge (IVAN SORVALL, Inc., Norwalk, Conn), and washed three times with 0.01 M NH_4Cl/NH_4OH, pH 7.2 buffer. The cell-free broth was saved for determination of G.E.H. activity. The pellet obtained was suspended in its same volume of the buffer (6-8 ml). The cell suspension was stored at -12°C until its use.

Cell Disruption

In order to release the cytoplasmic cell material, the harvested cells were disrupted in a BIOX-X-PRESS model X-25 (AB BIOX, SWEDEN). The press was immersed in an alcohol-water (60:40) bath, previously cooled to -25° to -30°C with commercial dry ice. The cell suspension was pipetted into the press, allowed to freeze and pressed five times at a pressure of about 2,000 kg per sq. cm. (28,000 psi). As the frozen cell pellet passed through the press, a pressure induced transition in the crystal structure changed the crystal from Ice I (ordinary ice) to Ice III. The shear stress, eddying and explosive decompression at flow disrupted the cells. After disruption, when not used immediately, the cells were stored at -12°C.

BIOX X-PRESS Efficiency

To check for press efficiency in disrupting the cells, samples were plated in Elliker's Lactic Agar (Elliker et al.,

1956), before and after pressing (see Appendix for medium composition). The ratio of the number counts of viable cells before and after disruption was calculated as percentage of efficiency of cell disruption by the BIOX X-PRESS.

Tributyrin Agar Well Method for G.E.H. Activity

To screen for G.E.H. activity the agar method of Oterholm and Ordal (1966) was modified. Filter sterilized tributyrin (0.3%) was emulsified 5 times in sterilized DIFCO BACTO agar (0.15%), pH 7.8, in a hand emulsion homogenizer (Logeman emulsifier) at about 4.1 MPa (600 psi), and poured in 65x15 mm Petri dishes to produce a certain thickness of agar layer in the Petri dish. Addition of 2.5 and 5.0 ml of melted agar-emulsion mixture produced 1 mm and 2 mm layers, respectively. Three 5.5 ± 0.3 mm wells were punched in the layer in each plate by using a No. 2 cork borer. The punched agar plugs were removed from the plates by suction with a Pasteur pipette. The wells were filled with 15 µl of test sample for 1 mm well depth and with 30 µl of the sample for 2 mm well depth. The plates were incubated at 30°C. After incubation of the plates for 12 to 48 h at 30°C G.E.H. activity was determined by measuring the diameter of zone of clearance around the wells. The larger the halo of hydrolysis the greater the lipolytic activity.

The original method of Oterholm and Ordal (1966) was modified as follows: (1) Only 1.5% agar suspension in water was utilized as the solid support for tributyrin and other lipids used in this study. Since growth of the microorganism was not desirable, MRS broth used in the original work was not added. (2) The tributyrin-agar mixture was emulsified by a hand homogenizer instead of the ultrasonic desintegrator originally employed.

The high sensitivity of the method permitted detection of G.E.H. activity even in microorganisms weakly lipolytic. Lawrence et al. (1967a) reported a similar G.E.H. assay to be almost 90 times more sensitive by substituting butterfat with tributyrin.

A prolonged contact of fat and lipase in a food product is likely during storage. Hence, a weakly lipolytic enzyme may bear significance as in storage of butter or cheese ripening.

Influence of Medium Composition and Incubation Temperature on G.E.H. Production by Streptococcus thermophilus

To study the influence of these two variables a strain of Streptococcus thermophilus was selected. The criteria for selection were: relatively highest G.E.H. activity along with fast and high cell production. The strain ST-9 was found to satisfy these criteria. This microorganism was maintained as indicated before and was frequently tested

microscopically for contamination.

The influence of medium composition and incubation temperature was studied by growing the culture at constant pH 6.0 in a 7.0 l Modular Microferm Bench Top Fermenter (NEW BRUNSWICK SCIENTIFIC CO., Inc., Edison, NJ) connected for constant pH control to a VIRTIS Digital pH Controller model 43 DPH CR which activated a VIRTIS pH Pump Module 43 PH-PE (THE VIRTIS COMPANY, Gardiner, NY). Whenever the pH of the culture medium fell below 6.0, the system introduced appropriate quantity of 1 N NaOH to keep the pH constant at 6.0-6.05.

The different conditions for testing G.E.H. production are presented in Table 3.

Incubation and Cell Harvesting

Twenty five milliliters of an 18 h Streptococcus thermophilus ST-9 culture in Hogg and Jago medium were aseptically added to 5 l of sterilized medium in the fermenter jar. The pH-meter electrode, after standardization, was chemically sterilized by wiping it respectively with 5% phenol and 70% ethanol. The appropriate incubation temperature was set and the pH of the medium was regulated to stay constant at 6.0. The culturing time generally ranged from 12 to 16 h. During incubation of the synthetic medium under pH stat condition, an uptake of 1 M NaOH reached 450-500 ml indicating utilization of 80-90% of the total lactose present. However, the volume of 1 M NaOH to keep the pH constant in the milk medium was in the range of 600-650 ml.

Table 3. Different media and growth temperature for testing G.E.H. production by Streptococcus thermophilus at constant pH 6.0

Culture medium	Incubation temperature (°C)
Hogg and Jago basal medium[a]	28
Hogg and Jago basal medium	37
Hogg and Jago basal medium	44
Hogg and Jago basal medium + 0.2% casein	37
Hogg and Jago basal medium + 1% butter oil	37
Hogg and Jago basal medium + 10% cream (10% fat)	37
Milk (3.4% fat)	37

[a]Lactose (1%) was added to Hogg and Jago basal medium

After growth the cells were harvested in a SORVALL Superspeed RC 2 B Automatic Refrigerated Centrifuge (IVAN SORVALL, Inc.) at 4°C and 13,000 xg. The cells were washed three times with 0.01 M NH_4Cl/NH_4OH, pH 7.2, buffer. The washed cell pellet was dispersed in the original volume of the buffer (25-30 ml). If not used immediately the harvested cells were stored at -12°C.

Cell Disruption and Fractionation

The cells were disrupted in a BIOX X-PRESS as described previously (page 35). Three kinds of cellular material were examined for G.E.H. activity:

a. The resting cells which did not undergo disruption treatment
b. The debris, obtained by centrifugation of the disrupted cells at 48000 xg and dispersed in the original volume of the buffer (3-5 ml)
c. The cell extract, the supernatant from step b.

Dry Cell Weight Determination

G.E.H. activity in this phase of the experiment was determined as a function of the dry solids present in the resting cell suspension. Approximately 1 g of the suspension was weighed accurately into a previously dried and tared aluminum dish. The sample was dried for 4 h in a vacuum oven at 100-105°C under 25 in of vacuum. The dried sample was cooled in a desiccator and weighed again. The

remaining solids were calculated as percentage of dry cell weight.

Silica Gel Assay for G.E.H. Activity

Samples of different type of cellular material were assayed by the silica gel chromatographic method described by Harper et al. (1956) with minor modifications (Chandan, 1962). In this method, the free fatty acids are chromatographically extracted from the enzyme-substrate mixture and titrated by a standard alcoholic KOH solution.

Materials and Reagents

a. Chromatographic columns, 38 mm in diameter and 230 mm in length, with a fritted glass disc sealed into a 34/28 standard taper joint.

b. Silicic acid, 100 mesh powder.

c. 2 M phosphate buffer, pH 6.5 (see Appendix).

d. Buffered silica gel slurry. Fifty grams of dry silicilic acid were thoroughly mixed with 30 ml of 2 M phosphate buffer and 200 ml of U.S.P. chloroform were added to form a homogeneous slurry. The slurry was stored in a tightly stoppered brown bottle under refrigeration.

e. Eluant. Five per cent n-butanol in chloroform (v/v).

f. Titrating solution. 0.01 N ethanolic KOH, standardized by potassium hydrogen phthalat.

g. 0.1% Phenol red indicator solution. 100 mg of phenol red were ground in 0.1 ml of 0.1 N KOH and made to 100 ml with absolute ethanol.

h. 20% (v/v) sulfuric acid solution.

i. Substrate. Ten grams of gum arabic were dissolved in 100 ml of hot water and 90 ml of this solution (at 60-65^{o}C) was mixed with 10 ml of tributyrin. The mixture was emulsified at 4.1 MPa, in a hand homogenizer 5 times and the pH was adjusted to 7.8 by addition of 0.1 N NaOH.

Determination of Released Free Fatty Acids

One milliliter of a sample (resting cells, cell debris, and cell extract) was incubated with 5 ml of substrate at 30^{o}C for 2 h. Following incubation, the enzyme-substrate mixture was acidified to pH 1.8 to 2.0, with 0.3 ml of 20% sulfuric acid. This treatment stopped the reaction. Eighteen grams of silica gel were added and the mixture was thoroughly ground.

Chromatographic Column Preparation

The column was composed of two sections:

a. Bottom section. The column was attached to a 500 ml suction flask and a filter paper disc (WHATMAN No. 5) was placed on the fritted glass disc bottom of the column to prevent plugging by the silica gel particles. Buffered silica gel slurry (25-30 ml) was placed on the top of this section. Suction was applied in such a way that the flow of the liquid was enough to form a uniform bed.

b. Top section. The ground, silica gel-acidified lipolyzed sample was slurried with 50 ml of the eluant and the flocs formed were reduced to small particles by pressing them with a spatula. It was quantitatively transferred to the top of the column, and the flask containing the mixture was washed two more times with 50 ml each of the eluant. Suction was applied to give an eluate flow of approximately 30 ml/min.

Titration of the Extracted Free Fatty Acids

Phenol red indicator (0.2 ml) and 15 ml of absolute ethanol were added to the eluate. The free fatty acids were titrated by 0.01 N alcoholic KOH to a phenol red end point. A blank containing 1 ml of the sample at the moment of the chromatographic extraction was also titrated and its value used as a correction factor (indigenous fatty acid content) for calculating the real titer value of developed fatty acids.

G.E.H. Activity

The activity of the enzyme source was expressed as micromoles of free fatty acids (butyric acid) released/hour/gram of dry cell weight of the resting cell suspension.

Fundamentals of the Silica Gel Method

The method is based on the determination of fatty acids liberated by lipase from tributyrin. The triglyceride and the released fatty acids are extracted with a chloroform: butanol solution and the total acidity is determined by

titration with alcoholic KOH.

The method can be criticized on the grounds that the liberated fatty acid may inhibit lipase activity. However, a drop of no more than 0.8 pH units was observed during the present experiments. On the other hand, according to Brockerhoff and Jensen (1974) the method recovers quantitatively all free fatty acids, including butyric.

Isolation/Concentration of the Enzyme

In order to increase the concentration of the enzyme and to obtain a certain degree of purification, the G.E.H. present in the cell extract was salted out at 10°C by dropwise addition of saturated ammonium sulfate to obtain 70% saturation.

After precipitation, the suspension was centrifuged at 48,000 xg and the pellet was dissolved in 4-5 ml of 0.01 M NH_4Cl/NH_4OH pH 7.2 buffer. The concentrated material was dialyzed in a cellulose acetate dialysis tubing with a M.W. cut off of 12,000-14,000 daltons. The sealed tubing was placed in cold distilled water, moderately stirred and kept at 4°C for 24 h. The water was replaced each 8 h. The dialyzed sample was kept under refrigeration.

Effect of Several Factors on G.E.H. Activity

Table 4 outlines the time-temperature relationship at which the concentrated enzyme preparation was tested concerning stability. Table 5 outlines the different tests

and conditions to determine the effect of several variables on G.E.H. activity of Streptococcus thermophilus.

Methods Utilized to Test for G.E.H. Activity

The tributyrin agar well method previously described (page 36) was complemented with the pH stat method in this phase of the study.

pH Stat Method

The pH stat equipment was composed of four basic components:

a. Titrator (E526 METROHM HERISAU, Switzerland).
b. Motor Driven Piston Burette (E 525 METROHM HERISAU)
c. Potentiometer Recorder (SERVOGOR 210, Austria)
d. Constant Temperature Circulator Model 80 (Fisher Scientific Company, Pittsburgh, PA)

Five milliliters of the substrate (Table 6) were placed into the reaction vessel of the equipment and adjusted to a specific temperature. The dialyzed G.E.H. extract (0.5 ml) was added and the emulsion was automatically adjusted to the desired pH. As a result of the G.E.H. action, free fatty acids were released in the reaction mixture. Consequently, the pH dropped below the preset value. However, it was automatically returned to the preset value by addition of 0.02 N NaOH solution. The volume of alkali utilized was registered in the potentiometric recorder chart. The

Table 4. Outline for determination of stability of G.E.H. of *Streptococcus thermophilus*

Temperature (°C)	Time
- 15	0 h, 1, and 7 days
4	0 h, 1 and 7 days
37	0 h, 6 h, 18 h, 1, 2 and 7 days
45	0 h, 6 h, 18 h, 1, 2 and 7 days
65	30 min
100	2 min
121	15 min

Table 5. Outline of tests and conditions to determine the effect of several variables on G.E.H. activity of *Streptococcus thermophilus*

	Conditions				
Variable	Temperature (°C)	pH[c]	Enzyme concentration	Salts added[d]	Assay method and substrate[e]
Temperature	5[a], 25, 30, 35, 40, 45, 50[b], 65[b] 70[b]		-	-	pH stat and tributyrin agar well methods
pH	45	4[a], 5[a], 6[a], 7, 8, 9, 10, 11[b]	-	-	pH stat and tributyrin agar well methods
Enzyme concentration	45	9.0	Enzyme preparation was diluted with the ammonium buffer to yield 50, 25, 10, 5 and 2.5% conc.	-	pH stat and tributyrin agar well methods
Salts added	45	9.0	-	Sodium desoxycholate Sodium taurocholate Calcium chloride	pH stat and tributyrin agar well methods
Substrate	45	9.0	-	-	Corn oil, olive oil, soybean oil, butteroil, tributyrin

[a]Only tested by the tributyrin agar well method
[b]Only tested by the pH stat method
[c]Buffers utilized to control the pH are listed in the appendix
[d]Salts were added at a concentration of 0.5, 1.0, 2.0, 5.0, and 10 micromoles/ml of substrate
[e]Except for the variable substrate, tributyrin was the only substrate utilized

Table 6. Tributyrin-gum arabic emulsion for G.E.H. activity determination by pH stat method[a]

Gum arabic	10 g
Lipid source[b]	10 ml
Distilled water q.s.p.	100 ml

[a]The lipid source was added to the aqueous gum arabic solution and the emulsion was homogenized in a hand homogenizer for 5 times at temperature of 60-65°C. The pH was adjusted to that required by adding 0.1 N NaOH.

[b]For lipid source see Table 5 (substrate).

time for the test varied between 7-10 min, and the interval of time between the second and the seventh minute of reaction was used in the calculation of G.E.H. activity. G.E.H. activity was expressed as micromoles of butyric acid released/min/mg of protein in the enzyme preparation. A blank with the autoclaved enzyme ($121^{o}C$/15 min) was assayed and the result used as a correction factor.

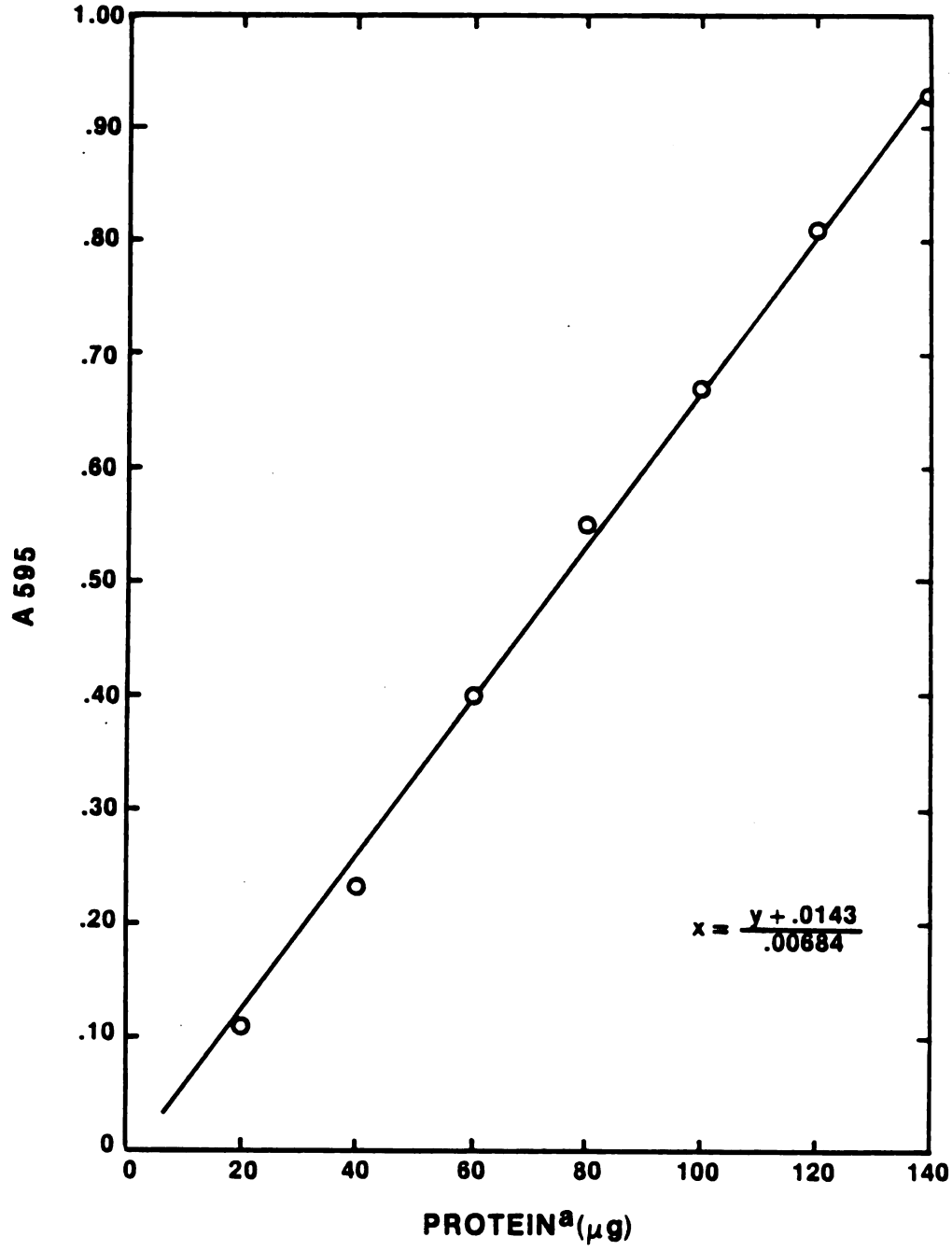
According to Brockerhoff and Jensen (1974), the pH stat method is valuable and indispensable in investigations of lipase kinetics as initial velocities can be determined directly. Additionally no extraction of the liberated acids is necessary, and no manual adjustment of pH is involved. According to Parry et al. (1966) this method is considerably more sensitive than the silica gel method. The method presents some disadvantages. It is difficult to measure lipase activity at pH <7.0, probably due to the incomplete titration of fatty acids with low dissociation constants. Also, the presence of buffer or substances as peptones interfere with the titration. In this particular experiment these disadvantages did not affect the assay since pH was 9.0 for most of the experiments, and butyric acid is completely dissociated at this pH.

Protein Determination

Protein was determined by the BIO-RAD Protein Assay Method (BIO-RAD Laboratories, Richmond, CA). This method

is based on the work of Bradford (1976). An acidic solution of Coomassie brilliant blue G 250 was used to bind protein. The principle of the method is that the absorbance maximum for the dye solution shifts from 465 nm to 595 nm following binding to protein (Reisner et al., 1975; Sedmak and Grossberg, 1977). A lyophilized bovine gamma globulin (BIO-RAD LABORATORIES) was utilized to prepare the standard protein solution. The absorbance of the protein dye mixture was read at 595 nm in a Spectronic 20 (BAUSCH & LOMB) after color development for 20 min. A reagent blank was used in the reference cuvette. (A typical standard curve is given in Fig. 1).

The data presented in this work are averages of 2-3 trials, run in duplicates.



[a]A lyophilized bovine gamma globulin was utilized to prepare the standard curve.

Figure 1. Standard curve for protein determination (BIO-RAD METHOD).

RESULTS AND DISCUSSION

Standardization of the Tributyrin Agar Well Method

In the first phase, an experiment was performed to determine an optimum thickness of the agar layer for the tributyrin agar well method. Oterholm and Ordal (1966) suggested an incubation time of 30 h for plates containing 1 mm agar layer. It can be inferred from their work that under these conditions, sufficient contrast between the medium and the zone of clearance around the wells was maintained in the agar tributyrin emulsion. However, in the present work a definite lack of contrast of the emulsion was observed after 12 h of incubation. Since the method is based on the clearance of the emulsion in the hydrolyzed area, no more differentiation was possible. To enhance resolution of the zone of clearance, 2 mm thick agar layer plates were tried. These plates kept the opacity up to 48 h of incubation, after which the contrast was decreased. Accordingly, the two mm layer was used in subsequent work.

The disagreement with the results of Oterholm and Ordal (1966) may possibly be ascribed to the degree of dispersion of fat globules and stability of the emulsion.

These authors used an ultrasonic disintegrator to prepare the emulsion, whereas a hand homogenizer was utilized

in the present work.

Efficiency of Cell Disruption

Efficiency of cell disruption by the BIO X-PRESS is shown in Table 7. These data show the counts of bacteria in suspension plated before and after disruption. It may be seen that by passage of the frozen cell suspension of Streptococcus thermophilus through the press for 5 times effected at least 98% breakage of the cells. In approximately 50% of the cases the disruption exceeded 99.99%. The results are in agreement with the equipment manufacturer claim of 99% cell disruption in the case of Streptocuccus faecalis, under similar conditions.

Screening for G.E.H. Activity of Streptocuccus thermophilus

Table 8 shows the results of G.E.H. activity of 32 disrupted cells cultures of Streptococcus thermophilus as determined by the tributyrin agar well method. It may be seen that the diameter of zone of clearance obtained with the positive strains varied between 5.91 and 8.90 mm. The average diameter of zone of clearance was 7.13 mm. The culture supernatant showed no measurable activity indicating the cell-bound location of the lipase in Streptococcus thermophilus. Results are in agreement with those of other workers (Carini et al., 1968; Carini, 1969; Angeles and Marth, 1971; Formisano et al., 1974; Brandl and Pfleger, 1976) who demonstrated the presence of tributyrinase activity in

Table 7. Percentage of Streptococcus thermophilus cells disrupted by freeze pressing at 200 MPa in BIOX X-PRESS

Culture strain	Plate count/ml[a]		
	Before pressing	After pressing	% Disruption
SF - 1	3.2×10^{10}	3.0×10^{6}	> 99.99
ST - 9	3.0×10^{9}	5.8×10^{7}	98.07
SF - 3	4.0×10^{9}	2.4×10^{6}	99.94
19987	2.6×10^{9}	2.7×10^{7}	98.96
ST - 9	4.6×10^{10}	3.8×10^{6}	> 99.99
ST - 9	2.4×10^{10}	9.5×10^{5}	> 99.99

[a]Elliker's medium was used. For medium composition see Appendix p. 98.

Table 8. Range of G.E.H. activity of disrupted cells of various strains of Streptococcus thermophilus assayed in 2 mm layer of modified pH 7.8 tributyrin agar

Diameter of zone of hydrolysis (mm)[a] observed with various strains					
No hydrolysis	5.91 - 6.50	6.5 - 7.10	7.11 - 7.70	7.71 - 8.30	8.31 - 8.90
ST - AH	EB - 8	9353	MC	4074	SFi - 3
IS	6031	CNRZ 391	CNRZ 406	14485	SFi - 1
	ST - 3	ST - 8	6069	3641	
	4109	CNRZ 404	19258	6097	
	P 371	MLT 33		ST - 9	
		7024			
		19987			
		ST - 7			
		7132			
		DS			
		YB - ST			
		L - 225			
		ST - 4			
		STs			

[a]Diameter of zone of hydrolysis includes well diameter

Streptococcus thermophilus. Additionally several reports have shown the cell bound location of lipase in lactic acid bacteria (Oterholm et al., 1968; Chandan et al., 1969; Formisano et al., 1974). In the present study, 30 cultures out of 32 displayed G.E.H. activity which is in disagreement with the findings of Chander and Chebbi (1972). They detected no G.E.H. activity toward tributyrin in all eight cultures of Streptococcus thermophilus they tested. Since they did not disrupt the cells, it is probable that the microbial cells did not release the enzyme into the medium.

To determine further lipase production and activity under different conditions, a strain of Streptococcus thermophilus, ST-9, was selected from the screening study of the thirty two strains. The selection was based on rapidity of growth and production of relatively maximum lipase under the conditions of the test.

Influence of Growth Temperature on G.E.H. Production by Streptococcus thermophilus

Table 9 shows the effect of growth temperature on the lipase activity of whole cells and the fractions. Lipase activity of intact whole cells reached a plateau at 37-44^{o}C. When compared to 37-44^{o}C, the lipase production of the whole cells grown at 28^{o}C represented 87% of that obtained at higher temperature. Optimum temperature for the total G.E.H. production of the cells (sum of cell debris fraction

Table 9. Influence of growth temperature on G.E.H. production by *Streptococcus thermophilus* (silica gel assay)

Growth Temperature (°C)	G.E.H. activity[a]				
	Whole resting cells	Cell debris fraction	Cell extraction fraction	Cell debris fraction[b] + cell extract fraction	Relative G.E.H. activity (%)
28	183	164	124	288	97
37	211	121	130	251	85
44	211	148	149	297	100

[a]Micromoles of butyric acid released/hour/gram of dry cell weight under the assay conditions.

[b]Considered as the total G.E.H. activity of the cell.

+ cell extract) was obtained at 44°C (100%) followed by 28°C (96%) and 37°C (85%). Since the sum total G.E.H. activity of the two cell fractions was greater than that of the whole cell suspension, it appears that the lipase is not totally available at the cell surface. Possibly some lipase may be located in the more internal layers of the cell membrane or even in the cytoplasm.

In general incubation temperature did not appear to influence substantially the G.E.H. production by the microorganism.

The optimum temperature of 44°C for lipase production by Streptococcus thermophilus is in agreement with those of some workers who reported maximum G.E.H. production by certain microorganisms at their optimum growth temperature (Mates and Sudakevitz, 1973; Jonsson and Sniff, 1974; Chander et al., 1980). However, other workers have found that the amount of lipase production in some microorganisms is higher at temperatures below their optimum growth temperature (Nashif and Nelson, 1953a; Lawrence, 1967). Apparently, at lower temperatures the action of inherent proteases might be reduced to observe higher lipase activity. At 37°C lipase production was lower. It may be conjectured that proteases active toward the lipolytic enzyme protein may be more active at 37°C than at 28° or 44°C.

Effect of Adding Lipids to the Growth Medium on G.E.H. Production by Streptococcus thermophilus

Preliminary assay trials using the agar diffusion method indicated little or no clearance zones when butter oil and other natural lipids were substituted by tributyrin. Therefore, silica gel chromatography was employed. The results are shown in Table 10. Lipase activity of whole cells appeared to be inhibited by the addition of lipids, when compared to the medium containing no lipids. However, the total G.E.H. activity of the cells (cell debris + cell extract fractions) grown with corn oil and soybean oil showed increase of 21% and 39%, respectively, when compared with that of the control containing no lipids. Butter oil was observed to depress lipase production in the three types of cellular material (whole resting cells by 35%; cell debris fraction by 17% and cell extract fraction by 40%). Total G.E.H. production was reduced by 29%. Hence, the adaptative character of G.E.H. of the microorganism depended on the lipid added. These observations are in agreement with several reports showing inhibition of lipase production by lipids, as in Pseudomonas fragi (Smith and Alford, 1966); Penicillium roqueforti (Eitenmiller et al., 1970); Rhizopus stolonifer (Chander et al., 1981). Stimulation of lipase production in presence of higher fatty acid or oil in the medium by Geotrichum candidum has been reported by Iwai et al. (1973).

Table 10. Influence of addition of different lipids to growth medium on G.E.H. production by Streptococcus thermophilus (silica gel assay)

Growth medium	G.E.H. activity[a]				
	Whole resting cells	Cell debris fraction	Cell extract fraction	Cell debris fraction[b] + cell extract fraction	Relative G.E.H. activity (%)
Hogg and Jago medium	211	121	130	251	100
With 1% corn oil	179	164	140	304	121
With 1% soybean oil	169	167	182	349	139
With 1% butter-oil	138	101	78	179	71

[a]Micromoles of butyric acid released/hour/gram of dry cell weight under the assay conditions.

[b]Considered as the total G.E.H. activity of the cell.

Influence of the Addition of 0.2% Casein to the Growth Medium on G.E.H. Production by Streptococcus thermophilus.

Results are presented in Table 11. Total G.E.H. production (cell debris + cell extract fractions) decreased 12% in the medium containing casein. Chandan et al. (1969) found casein to be slightly inhibitory to cellular lipase production by Streptococcus cremoris, Streptococcus diacetilactis, Streptococcus durans, Streptococcus lactis, Leuconostoc citrovorum and Propionibacterium shermanii. However, Streptococcus thermophilus was not studied by these workers.

Comparative G.E.H. Production by Streptococcus thermophilus in Hogg and Jago Medium; Hogg and Jago Medium Containing Cream, and Milk

Table 12 shows that growth in milk strongly inhibited lipase production when compared with the Hogg and Jago medium. Inversely, when cream was added to the synthetic medium, there was an increase in G.E.H. production. As observed earlier in this work, casein and butterfat present in substantial amounts in cream, were inhibitory to G.E.H. production by Streptococcus thermophilus when added individually to the Hogg and Jago medium. Accordingly, the stimulatory effect observed with cream may be ascribed to cream components other than butterfat and casein.

Table 11. Influence of addition of 0.2% casein to growth medium on G.E.H. production by *Streptococcus thermophilus* (silica gel assay)

Growth medium	G.E.H. activity[a]				
	Whole resting cells	Cell debris fraction	Cell extract fraction	Cell debris fraction[b] + cell extract fraction	Relative G.E.H. activity (%)
Hogg and Jago medium	211	121	130	251	100
Hog and Jago medium + 0.2% of casein	179	127	94	221	88

[a]Micromoles of butyric acid released/hour/gram of dry cell weight under the assay conditions.

[b]Considered as the total G.E.H. activity of the cell.

Table 12. Comparative G.E.H. production by Streptococcus thermophilus in Hogg and Jago medium, Hogg and Jago medium + cream (10% fat) and milk (silica gel assay)

Growth medium	G.E.H. activity[a]				
	Whole resting cells	Cell debris fraction	Cell extract fraction	Cell debris fraction[b] + cell extract fraction	Relative G.E.H. activity (%)
Hogg and Jago medium	211	121	130	251	100
Hogg and Jago medium + 10% cream (10% fat)	158	196	123	319	127
Milk	117	79	69	148	59

[a]Micromoles of butyric acid released/hour/gram of dry cell weight under the assay conditions.

[b]Considered as the total G.E.H. activity of the cells.

Enzyme Stability

Results of lipase stability under different conditions of storage and heat treatment are presented in Table 13. The concentrated enzyme preparation was stable at high temperatures of storage (37°C and 45°C) even for relatively long periods of time, when compared with the sample at zero hour storage time. The optimum temperature for G.E.H. activity of Streptococcus thermophilus was 45°C. At storage temperatures of 4°C and -15°C, some loss of activity was noticed. It was more pronounced at 4°C (23% loss) than at -15°C (17% loss), at the end of one week of storage.

When the enzyme was submitted to heat treatment, a definite decrease in activity was observed at 65°C for 30 min. with only 27% of the original activity being retained. The results show that the lipase of Streptococcus thermophilus is partially resistant to pasteurization treatment. After boiling for 2 min and sterilization treatment of 121°C for 15 min the lipase was completely inactivated.

Various microbial lipases vary in their resistance to heat treatment. Csiszar and Romlehner-Bakas (1956) reported that a lipase from Pseudomonas fluorescens was inactivated at 100°C, but retained 32% of activity when heated at 75°C for 10 min. Pinheiro et al. (1965) obtained inactivation of the lipase of Pseudomonas fluorescens at pasteurization temperature (63°C for 30 min). However, Anderson et al. (1979) described a Pseudomonas fluorescens lipase that was

Table 13. Stability of G.E.H. of Streptococcus thermophilus as a function of storage temperature and time

Temperature	Time of storage	% residual activity
-15°C	24 h	100
	1 wk	83
4°C	24 h	90
	1 wk	77
37°C	6 h	100
	18 h	100
	24 h	100
	48 h	100
	1 wk	100
45°C	6 h	100
	18 h	100
	24 h	100
	48 h	100
	1 wk	100
65°C	30 min	27
Boiling	2 min	0
121°C	15 min	0

inactivated only when it was treated at 140°C for 3.6 min in nutrient broth or at 140°C for 2 min in skim milk. Results for Streptococcus thermophilus lipase showed some similarity with those of Chander et al. (1979a). They found that treatments of 60°C for 10 min and 90°C for 10 min inactivated a lipase of Streptococcus faecalis by 40% and 100%, respectively. Additionally, the enzyme was more resistant than a lipase from Lactobacillus brevis, which was completely inactivated either by heat treatment of 62.8°C for 30 min or 71.7°C for 16 sec (Chander et al., 1973).

According to Anderson et al. (1979) factors involved in the heat resistance of an enzyme are: primary structure of the enzyme molecule and specific components such as polysaccharides and divalent cations capable of stabilizing the molecule. Calcium ions have been shown to increase the thermal stability of a Pseudomonas protease (Barach et al., 1976). Also, a high content of hydrophobic amino acids in the enzyme molecule, disulfide bridges and other bonds are suggested to play an important role in stabilizing the molecule (Anderson et al., 1979; Liu et al., 1973; Liu et al., 1977).

Streptococcus thermophilus lipase differed from other microbial lipases in its behavior at different storage temperatures. Many microbial lipases remain stable at low and subfreezing temperatures and lose activity at room or higher temperatures (Nashif and Nelson, 1953a; Finkelstein

et al., 1970; Chander et al., 1979). According to Frieden (1971), it could be the result of cold inactivation for which the mechanism of inactivation may be the dissociation of the monomer into subunits. The dissociated and inactive enzyme may be slowly converted to a different form which, although reassociation may occur, is still inactive after rewarming. Additionally, according to Cooper (1977) freezing and thawing of some protein solutions can decrease stability.

Effect of Temperature on G.E.H. Activity of Streptococcus thermophilus

G.E.H. activity toward tributyrin was determined at different temperatures. A concentrated enzyme preparation was used in this work. The assay methods employed were pH stat and tributyrin agar well methods. Figure 2 shows the results. The optimum temperature for G.E.H. activity of Streptococcus thermophilus was 45°C when determined by the two methods. Above 45°C the thermal inactivation of the enzyme increased and a rapid decrease in activity was observed.

Temperatures below 45°C also decreased G.E.H. activity, but the effect was less pronounced than for the similar increase in temperature above 45°C. For example, at 35°C and 55°C, the micromoles of free fatty acid released/min/mg of protein were 2.88 and 2.5, respectively by the pH stat method. G.E.H. activity at 5°C was very low as determined by the tributyrin agar well method.

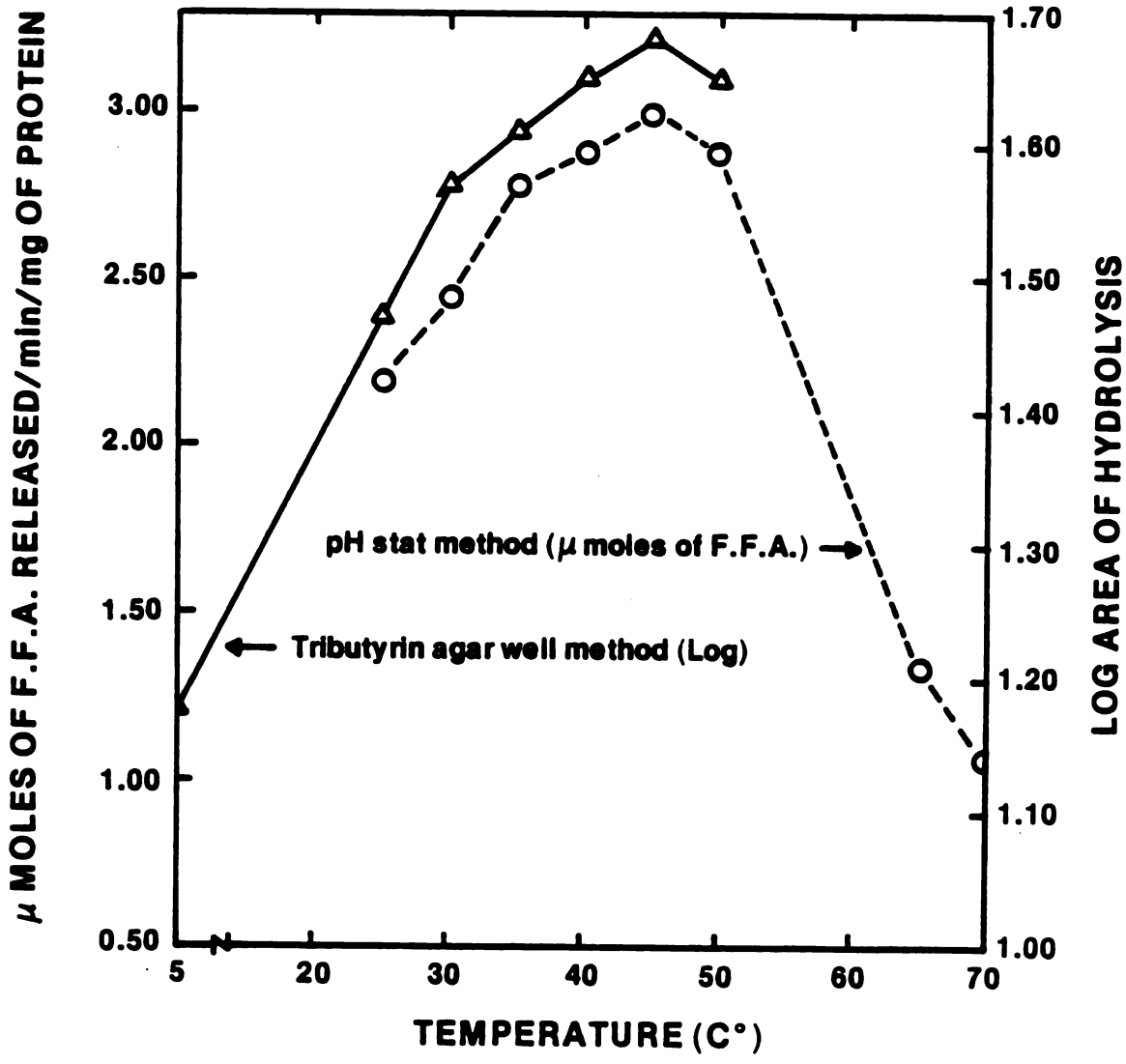


Figure 2. Effect of temperature on G.E.H. activity of Streptococcus thermophilus, ST-9 strain, at pH 9.0, toward tributyrin, as determined by pH stat and tributyrin agar well methods.

Most of the microbial lipases have been reported to have their optima within the range of 30-40°C (Hugo and Beveridge, 1962; Khan et al., 1967; Eitenmiller et al., 1970; Tsujisaka et al., 1973). However, some workers have reported higher temperatures for microbial lipase activity. Among microorganisms utilized in dairy products, Umemoto et al. (1968) reported 45°C as the optimum temperature for G.E.H. activity of Streptococcus diacetilactis while Oterholm et al. (1970) found 47°C as the optimum temperature for a purified G.E.H. of Propionibacterium shermanii.

Oterholm et al. (1970) suggested that the observed difference between the optimum temperature of the lipase of Propionibacterium shermanii (47°C) and most other microbial lipases was probably due to differences in the assay conditions used. The long incubation periods used by various workers and the data based on initial velocity for Propionibacterium shermanii may cause the discrepancy in the measurement of the optimum temperature. Thermal destruction of an enzyme is generally progressive. The shape of the curve and the optimum temperature may be expected to be time dependent. However, in the case of Streptococcus thermophilus, the G.E.H. assayed showed the same optimum temperature, by the pH stat method (initial velocity studies) and by the tributyrin agar well method, employing long incubation period.

Effect of pH on the G.E.H. of Streptococcus thermophilus

The pH optimum of the G.E.H. of Streptococcus thermophilus was determined using tributyrin as a substrate. A concentrated enzyme preparation was used employing the pH stat and tributyrin agar well methods. Results given in Figure 3 show that both methods gave the optimum pH of 9.0 for the G.E.H. activity. As the pH of the substrate decreased or increased in relation to the optimum, there was a decrease in the enzyme activity. At pH 11.0 and 4.0, only slight G.E.H. activity was displayed toward tributyrin. The G.E.H. remained fairly active at pH 10.

Umemoto et al. (1968) found that the optimum pH for G.E.H. activity of Lactobacillus casei, Lactobacillus plantarum, Lactobacillus helveticus and Streptococcus diacetilactis varied between 7 and 8, with a sharp decrease at pH 9. Carini (1969) found pH 8 to be the optimum for lipase of streptococci and lactobacilli. Chander et al. (1973) demonstrated that the optimum pH for G.E.H. activity of Lactobacillus brevis was 6.5. The present results however, are in agreement with some reports that showed pH 9.0 or >9.0 as the optimum for G.E.H. activity of certain microorganisms (Hugo and Beveridge, 1962; Shahani et al., 1964; Jonsson and Snigg, 1974).

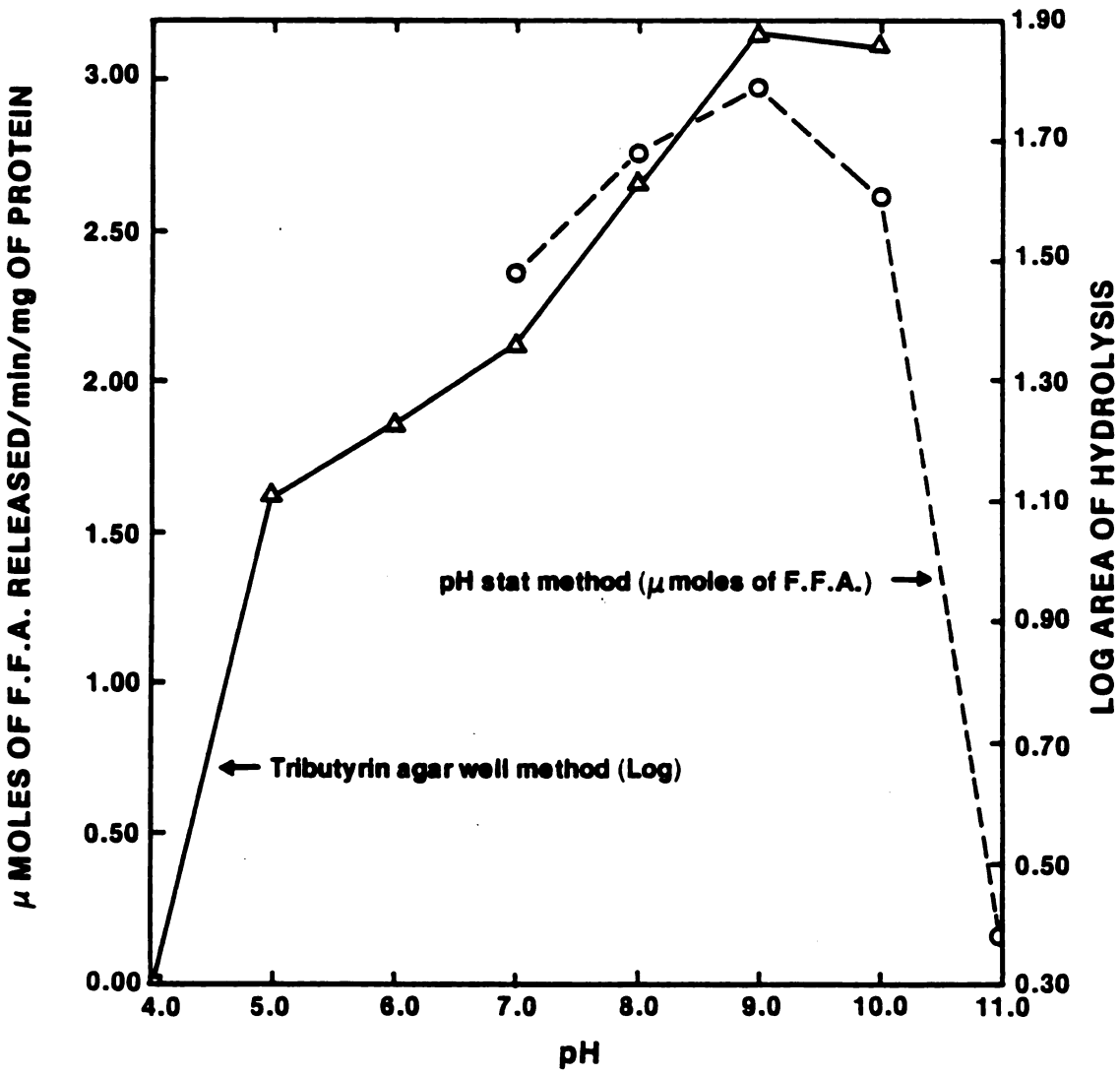


Figure 3. Effect of pH on G.E.H. activity of Streptococcus thermophilus, ST-9 strain, at 45°C, toward tributyrin as determined by pH stat and tributyrin agar well methods.

Effect of Addition of Bile Salts and Calcium Chloride to the Assay Emulsion on the G.E.H. Activity

Effect of the addition of $CaCl_2$, sodium desoxycholate, and sodium taurocholate on G.E.H. activity of Streptococcus thermophilus was studied by the pH stat and the tributyrin agar well methods. A concentrate enzyme preparation was used in this work. A control containing no salt was also assayed. Figure 4 shows that the addition of 10 and 50 micromoles of $CaCl_2$/ml of substrate increased the initial rate of reaction, as determined by the pH stat method. Addition of smaller amounts did not appear to influence the G.E.H. activity. A decrease in the G.E.H. activity was observed at all calcium chloride concentrations by the tributyrin agar well method, when compared with the control.

It appears that $CaCl_2$ at certain concentrations can stimulate the initial rate of reaction but the effect is inhibitory when long periods of incubation are necessary.

Literature reports show both stimulatory and inhibitory effects of $CaCl_2$ on G.E.H. activity. It appears however, that the salt concentration, the assay method and the incubation time play an important role in the effect of the salt.

The effect of addition of different concentrations of sodium desoxycholate to the substrate is shown in Figure 5.

Inhibition was observed at all salt concentrations when the tributyrin agar well method was employed. When pH stat method was employed a stimulatory effect was observed at concentrations of 1 and 2 micromoles of salt/ml of substrate. Higher concentrations were found to inhibit the enzyme activity.

The effect of sodium taurocholate addition to the substrate on G.E.H. activity of Streptococcus thermophilus is shown in Figure 6. The salt was found to be inhibitory to G.E.H. activity by both the methods. This inhibition was directly proportional to the salt concentration.

The results are in agreement with those of Iwai et al. (1964), Finkelstein et al. (1969), Saiki et al. (1969), and Oterholm et al. (1970). However, they disagree with reports of Nashif and Nelson (1953), Yamaguchi et al. (1973), and Chander et al. (1979), which found sodium taurocholate to be stimulatory to the G.E.H. activity of Pseudomonas fragi, Chromobacterium and Streptococcus faecalis, respectively. Many of the disagreements found in the literature relative to the influence of these salts on G.E.H. activity might be caused by differences in the methods and conditions employed in their work.

G.E.H. Activity of Streptococcus thermophilus Toward Different Lipids

This study was undertaken to determine the reactivity of G.E.H. of Streptococcus thermophilus. The rates of

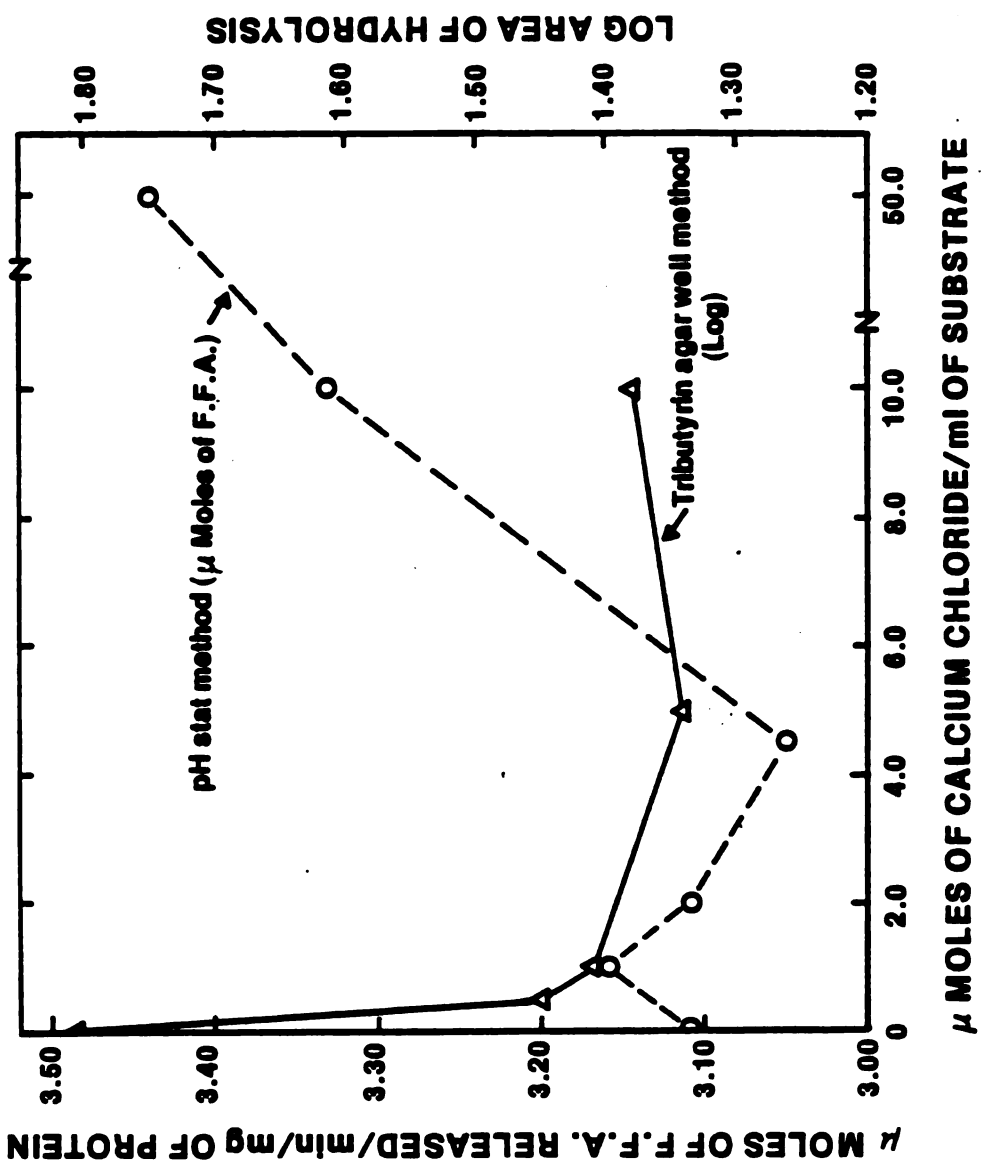


Figure 4. Effect of different concentrations of calcium chloride on G.E.H. activity of Streptococcus thermophilus, strain ST-9, at pH 9.0 and 45°C toward tributyrin as determined by pH stat and tributyrin agar well methods.

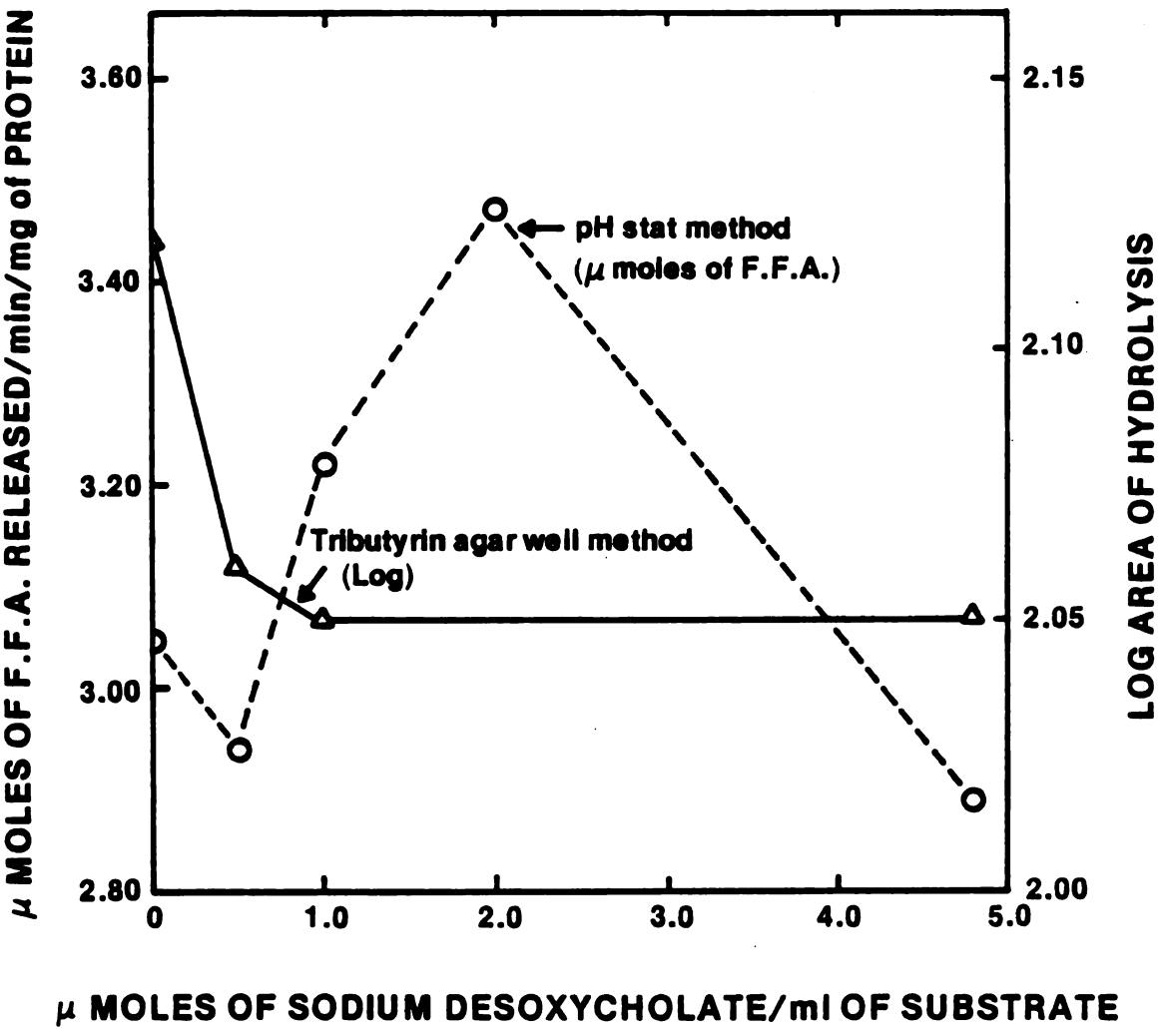


Figure 5. Effect of different concentrations of sodium desoxycholate on G.E.H. activity of Streptococcus thermophilus, ST-9 strain, at pH 9.0 and 45°C toward tributyrin as determined by pH stat and tributyrin agar well methods.

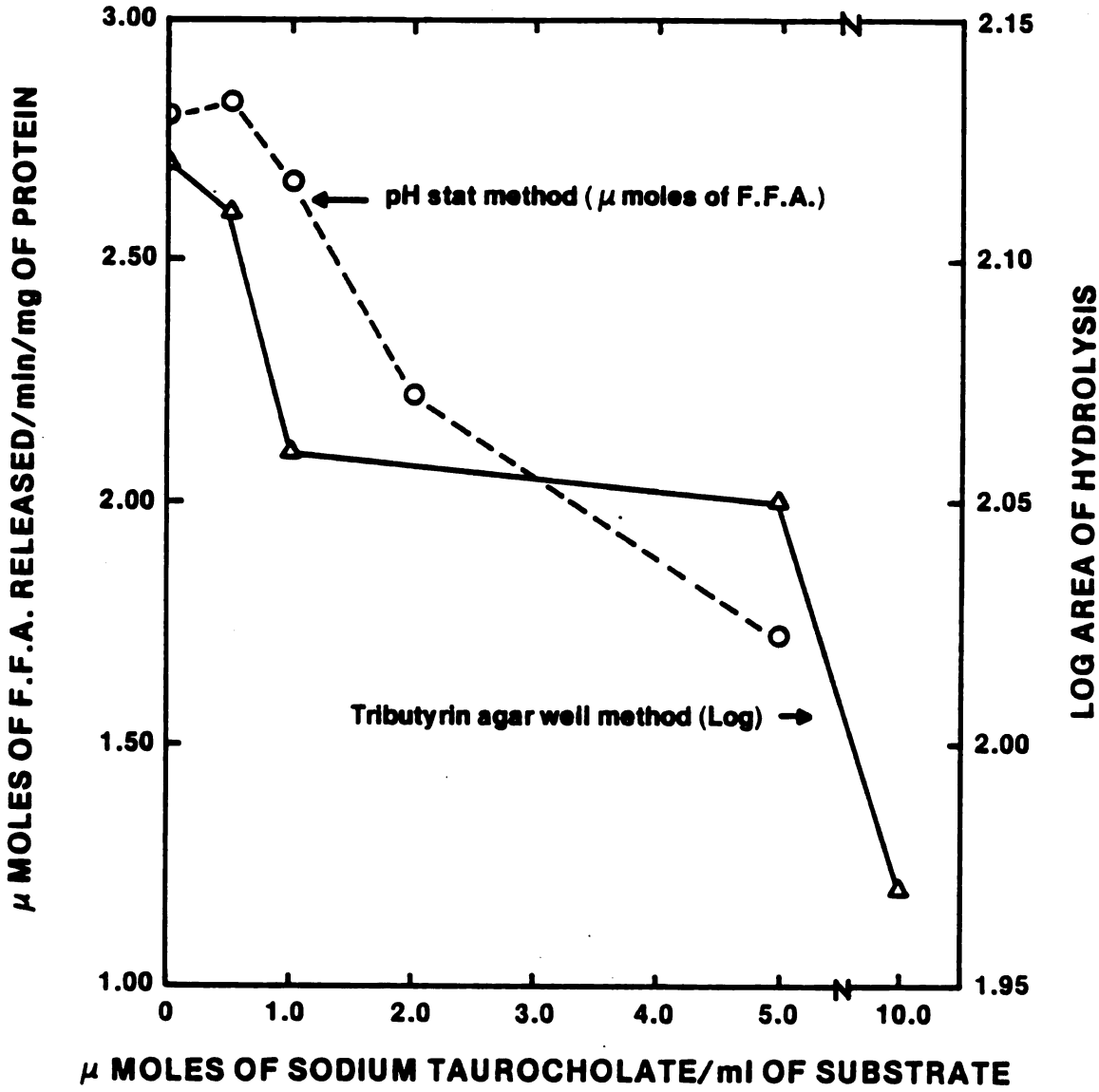


Figure 6. Effect of different concentrations of sodium taurocholate on G.E.H. activity of Streptococcus thermophilus, ST-9 strain, at pH 9.0 and 45°C toward tributyrin as determined by pH stat and tributyrin agar well methods.

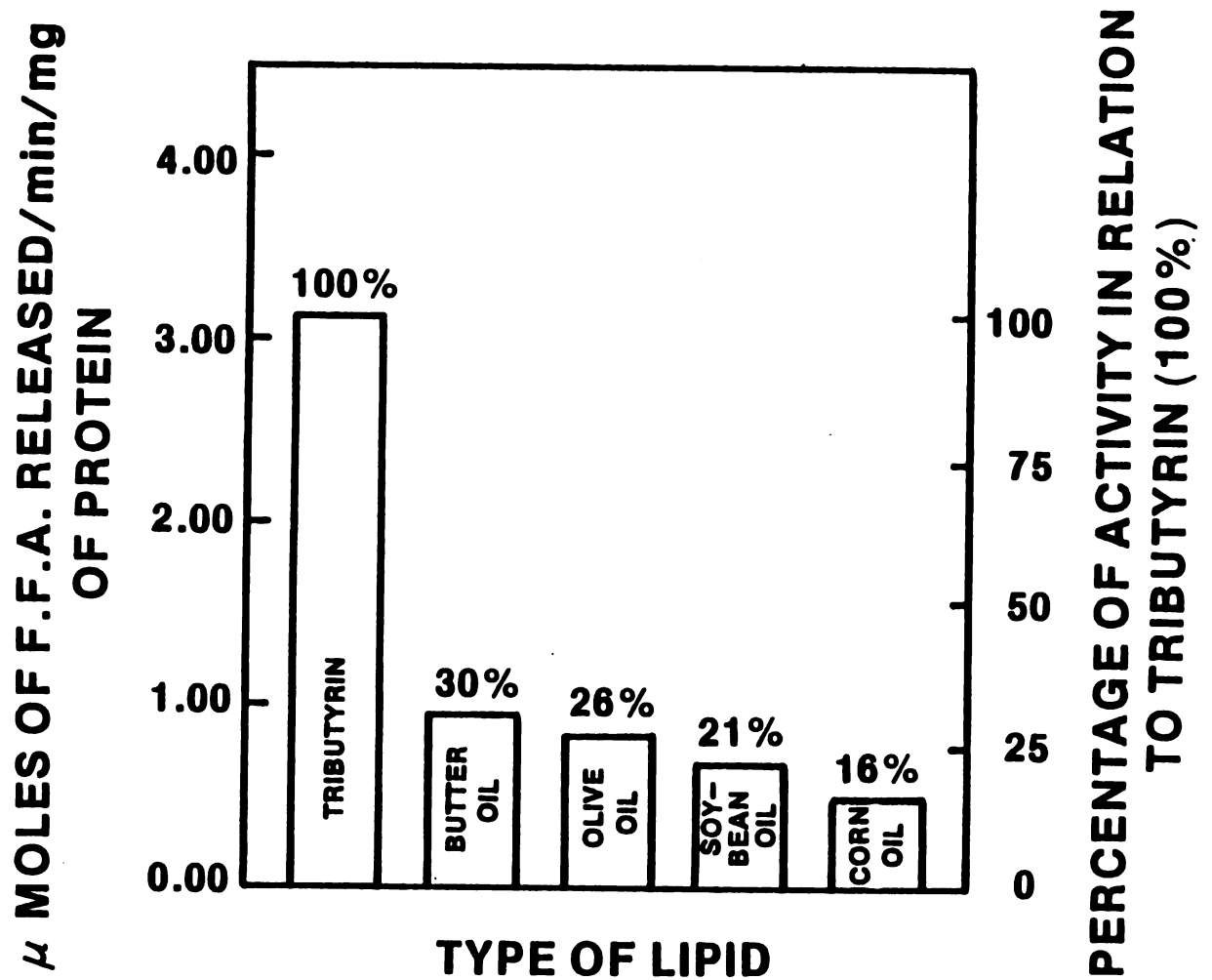


Figure 7. G.E.H. activity of Streptococcus thermophilus, strain ST-9, at pH 9.0 and 45°C, toward different lipids as determined by pH stat methods.

hydrolysis given in Figure 7 are all relative to the rate of enzyme activity on an emulsion of tributyrin. The relative rate of hydrolysis by the enzyme was highest for tributyrin followed by butter oil, olive oil, soybean oil and corn oil. The results corroborate the literature reports that microbial lipases preferentially hydrolyze triglycerides of short chain fatty acids (Umemoto et al., 1968; Oterholm et al., 1968; Eitenmiller et al., 1970; Oterholm et al., 1970; Angeles and Marth, 1971; Yamaguchi et al., 1973; Chander et al., 1973; Formisano et al., 1974; Chander et al., 1979). Microbial lipases of different specificity are also known. Khan et al. (1967) reported that extracellular lipase of Achromobacter lipolyticum hydrolyzed triolein more rapidly than tributyrin. Oi et al. (1969) found that an intracellular lipase of Rhizopus fungus hydrolyzed triolein more efficiently than tributyrin.

Effect of Enzyme Concentration on G.E.H. Activity of Streptococcus thermophilus

Figure 8 shows a linear relationship between the enzyme activity as a function of the enzyme concentration. Similar results were obtained by Oterholm et al. (1970) and Chander et al. (1979) when studying purified preparations of microbial lipases of Propionibacterium shermanii and Streptococcus faecalis, respectively.

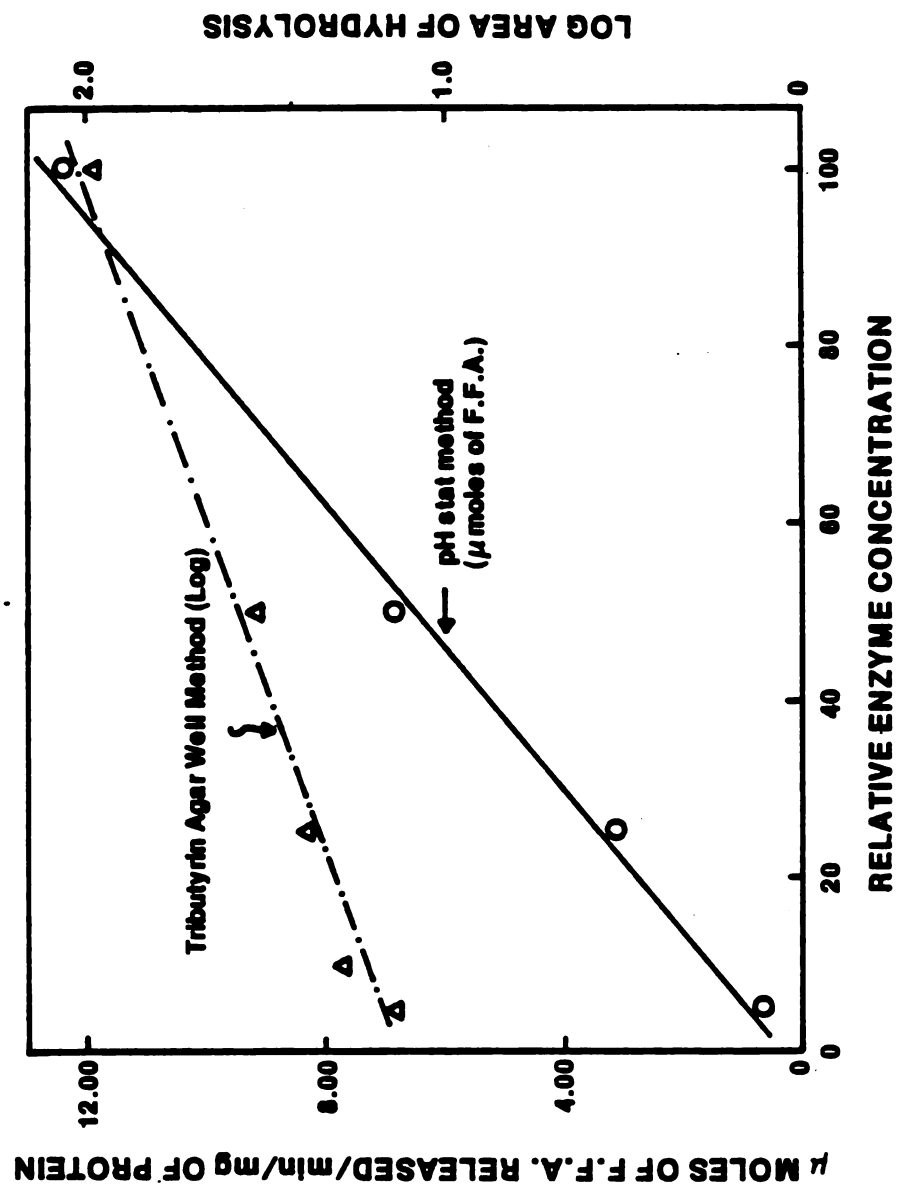


Figure 8. Effect of enzyme concentration on G.E.H. activity of Streptococcus thermophilus, strain ST-9, at pH 9.0 and 45°C as determined by pH stat and tributyrin agar well methods.

General Discussion

It appears that the conditions normally found in dairy products where Streptococcus thermophilus is employed are not conducive in relation to the optimum parameters observed for its lipase production and activity. Milk, butterfat and casein inhibited lipase production to variable degree. Lipase activity was partially inhibited at relatively low temperatures ($5^{o}C$) and at pH 4.0, the conditions normally found during the storage of yogurt. Additionally, butterfat was only weakly hydrolyzed by the concentrated enzyme preparation. In Swiss cheese the ripening process takes place at a pH of approximately 5.6 and temperatures of $20\text{-}24^{o}C$ for 3-6 weeks. After the first phase of ripening, the cheese is further held at $7.2^{o}C$ for 4-12 months. These pH and temperature conditions are more suitable for tangible G.E.H. activity. Also, the long period of ripening of some cheeses (Grana, 5-12 months, Cheddar, 4-12 months, and Swiss, 4-12 months) should allow for considerable lipolysis of milk fat. Consequently, Streptococcus thermophilus may not play an important role in the hydrolysis of fat during manufacture and storage of yogurt. However, the contribution of its G.E.H. on cheese ripening may relatively be fairly significant.

SUMMARY AND CONCLUSIONS

Thirty two strains of Streptococcus thermophilus were grown at 37°C for 18 hr in Hogg and Jago medium containing 1% lactose. The cells were harvested by centrifugation at 16600 xg for 10 min, washed three times in 0.01 M NH_4Cl/NH_4OH buffer, pH 7.2, and resuspended in minimum volume of the same buffer. Cells were disrupted by pressure in a BIOX X-PRESS at 200 MPa. Efficiency of cell disruption, calculated by determining the counts of bacteria plated before and after disruption, was showed to be at least 98%. The enzyme activity of the culture supernatant and disrupted cells was determined by a modified tributyrin agar well technique. Filter sterilized tributyrin (0.3%) was emulsified in 1.5% agar, pH 7.8, poured in 65x15 mm Petri dishes to produce 1 or 2 mm layer. Three 5.5 ± 0.3 mm wells were punched in the agar layer in each plate and filled with 15 µl for 1 mm well and with 30 µl for 2 mm well depth. The enzyme activity was determined by measuring the diameter of zone of clearance around the wells after incubation at 30°C for 12 to 48 hr. The zone diameter varied between 5.91 and 8.90 mm, with an average of 7.13 mm. All the cultures except two showed varying degree of activity against tributyrin. The culture supernatant showed no activity

indicating the cell bound location of the enzyme. Results showed that the plates containing 2 mm layer with 30 µl of inoculum gave relatively consistent contrast between the zone of hydrolysis and the surrounding opaque medium up to 48 hr of incubation. The plates with 1 mm depth agar layer incubated with 15 µl of inoculum produced zones of clearance more rapidly. However, the zones were less defined and after 12 hr of incubation a definite lack of contrast between the clearance zone and the surrounding medium was observed.

For further studies, the Streptococcus thermophilus strain ST-9 was selected, based on the glycerol ester hydrolase (G.E.H.) production and cell growth criteria. G.E.H. production, activity and stability under different experimental conditions were investigated. The culture was grown in 5-liters of synthetic medium at constant pH 6.0. A 7.5-liter capacity Microferm fermenter was used. The culture was grown without aeration. Agitation of the medium was at 60 RPM. Time of concluding fermenter run was determined by lack of NaOH uptake. Normally 450-500 ml of 1.0 N NaOH was used in each fermenter run. The centrifuged cells suspended in the buffer were disrupted in frozen state and centrifuged again at 48,000 xg for 10 min at $4^{o}C$. Cell debris fraction and the supernatant containing all extract were then examined for lipase activity. Also lipase activity of whole resting cells was determined for comparison with the activities of

the fractions obtained after their disruption. A linear relationship was observed when different enzyme concentrations were assayed by the two methods employed.

G.E.H. Production

In this phase of the study, the lipase activity was assayed by incubating the different kinds of cellular material separately with a tributyrin emulsion substrate at 30°C for 2 h. Following incubation, free butyric acid formed as a result of lipase action was separated in a silica gel column. The G.E.H. activity was expressed as μmoles of butyric acid released per hour by one gram of dry cell weight.

Effect of Growth Temperature

The G.E.H. activity of whole cells reached a plateau at 37°-44°C. The lipase activity of the whole cells grown at 28°C represented 87% of that obtained at higher temperatures. Optimum temperature for G.E.H. production of the cells (sum of cell debris fraction + cell extract fraction) was obtained at 44°C. At 28° and 37°C the enzyme production was only 96% and 85%, respectively, of the observed production at 44°C. The fractions showed appreciable higher lipase activity in relation to the whole cells indicating that lipase activity was partly masked in the whole cells.

Effect of Adding Lipids to the Growth Medium

Lipase activity of whole cells appeared to be inhibited by the addition of lipids, when compared to the medium containing no lipids. However, the total G.E.H. activity of

the cells grown with corn oil and soybean oil, showed an increase of 21% and 39%, respectively when compared with that of the control containing no lipids. Butter oil was observed to depress lipase production in the three types of cellular material (whole cells by 35%, cell debris fraction by 17% and cell extract fraction by 40%). Total G.E.H. production was decreased by 29%.

Effect of Addition of 0.2% Casein to the Growth Medium

Total G.E.H. activity decreased by 12% when the culture was grown in the medium containing casein, while 15% decrease was observed for the whole cells.

Comparative G.E.H. Production in Various Media

A substantially higher lipase production was observed in the synthetic medium as compared to whole milk. On the addition of cream to the synthetic medium an increase of 27% in G.E.H. production was noticed. In relation to the synthetic medium lipase production in milk was lower by 41%.

Enzyme Stability

Lipase stability was tested in a concentrated enzyme preparation. The enzyme was stored at several temperatures for varying periods of time (Table 4). The enzyme was stable when stored at 37° and 45°C for 7 days. At storage temperature of 4°C and -15°C a slight loss of activity was noticed. Heat treatment at 65°C for 30 min reduced lipase activity by 73%. After boiling for 2 min and sterilization treatment of 121°C/15 min, the lipase was completely

inactivated.

Effect of Several Variables on G.E.H. Activity of Streptococcus thermophilus

For this part of the study a concentrated enzyme was used and pH stat and tributyrin agar well methods were employed.

Effect of Temperature

The optimum temperature for G.E.H. activity was 45°C when determined by the two methods. Above 45°C, the thermal inactivation of the enzyme increased and a sharp decrease in the activity was observed. The G.E.H. activity was very low as determined by the tributyrin agar well method.

pH Optimum

Both methods gave the optimum pH of 9.0 for the G.E.H. activity. At pH 4.0 and 11.0, only slight G.E.H. was displayed toward tributyrin. At pH 10.0, the G.E.H. remained fairly active.

Effect of the Addition of Bile Salts and $CaCl_2$ to the Assay Emulsion

Addition of 10 and 50 µmoles of $CaCl_2$/ml of substrate increased the initial rate of reaction as determined by the pH-stat method. Addition of smaller amounts appeared not to influence the G.E.H. activity. A decrease in the G.E.H. activity was observed when $CaCl_2$ was added to the assay mixture for the tributyrin agar well method.

Sodium desoxycholate (1 and 2 μmoles/ml of substrate) was observed to be stimulatory to the G.E.H. activity when pH stat method was utilized. Higher concentrations were found to inhibit the enzyme. However an inhibition was observed at all the salt concentrations when the tributyrin agar well method was employed.

Sodium taurocholate was found to be inhibitory to the G.E.H. activity by both the methods. This inhibition was proportional to the salt concentration.

G.E.H. Activity Toward Different Lipids

The relative rate of hydrolysis by the enzyme was highest for tributyrin, followed by butter oil, olive oil, soybean oil and corn oil. The results show that the enzyme preferentially hydrolyzes triglycerides of short chain fatty acids.

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APPENDIX

Culture Media, Strains and Buffers

Culture Media

a. Hogg and Jago Basal Medium (Hogg and Jago, 1970)

Bacto-Tryptone	30 g
Bacto-Yeast Extract	10 g
Bacto-Beef Extract	2 g
KH_2PO_4	5 g
Distilled Water	1000 ml

pH before sterilization: 6.0

The basal medium was supplemented before sterilization with 1% of lactose and sterilized at 121°C/20 min.

b. Elliker's Lactic Agar (Elliker et al., 1956)

Bacto-Yeast Extract	5 g
Bacto-Tryptone	20 g
Gelatin	2.5 g
Dextrose	5 g
Lactose	5 g
Sucrose	5 g
Sodium Chloride	4 g
Sodium Acetate	1.5 g
Ascorbic Acid	0.5 g
Bacto-Agar	15 g
Distilled Water	1000 ml

pH before sterilization: 6.8

The medium was sterilized at 121°C/20 min.

c. Reconstituted Non Fat Dry Milk

10 g of nonfat dry milk was dissolved in 100 ml of distilled water and the medium was sterilized at 121°C/20 min.

Stains

a. Gram Stain (Hucker Modification) (Finegold et al., 1978)

Reagents

1. Stock Crystal Violet

Crystal violet, 85% dye	20 g
Ethanol, 95%	100 ml

2. Stock Oxalate Solution

Ammonium oxalate	1 g
Distilled Water	100 ml

Working solution: Dilute the stock crystal violet solution 1:10 with distilled water, and mix with 4 volumes of stock oxalate solution. Store in a glass stoppered bottle.

3. Gram Iodine Solution

Iodine crystals	1 g
Potassium iodide	2 g

Dissolve them completely in 5 ml of distilled water, then add:

Distilled water	240 ml
5% sodium bicarbonate solution	60 ml

Store in an amber glass bottle.

4. Decolorizer

Ethanol	250 ml
Acetone	250 ml

Store in a glass stoppered bottle.

5. Counterstain

Safranin O	2.5 g
Ethanol 95%	100 ml

Working solution: dilute stock safranin solution 1:10 with distilled water, and store in a glass stoppered bottle.

Buffers

a. 0.01 M NH_4Cl/NH_4OH, pH 7.2

A 0.01 M NH_4Cl solution was prepared and the pH adjusted to 7.2 with 1 M NH_4OH.

b. KH_2PO_4, pH 7.2 buffer (plate count)

Stock buffer

Thirty four grams of KH_2PO_4 were dissolved in 500 ml of distilled water and the pH was adjusted to 7.2 with 1 M sodium hydroxyde. The volume was made to 1000 ml.

Working solution: Dilute 1.25 ml of stock buffer solution to 1000 ml with distilled water. Sterilize in autoclave at $121^{o}C$/20 min.

c. 2 M Phosphate buffer pH 6.4 (silica Gel Chromatographic Method

1. 2 M KH_2PO_4 (27.2 g/100 ml)
2. 2 M K_2HPO_4 (34.8 g/100 ml)

Working solution: Add K_2HPO_4 solution to KH_2PO_4 solution until the pH of the mixed solution reaches 6.4.

Table A1. Composition of Buffers Used in the Determination of pH Optimum of G.E.H. Activity of *Streptococcus thermophilus*[a]

Buffer	Reagent	Milliliters of the Reagents used to obtain pH values						
		4.0	5.0	6.0	7.0	8.0	9.0	10.0
Citrate-	0.1 M citric acid	30.7	24.3	17.9	6.5	-	-	-
Phosphate	0.2 M K_2HPO_4	19.3	25.7	32.1	43.6	-	-	-
Tris-HCl	0.2 M trisaminomethane	-	-	-	-	50.0	50.0	-
	0.2 M HCl	-	-	-	-	26.8	5.0	-
Glycine-	0.2 M glycine	-	-	-	-	-	-	50.0
Sodium hydroxyde	0.2 M NaOH	-	-	-	-	-	-	32.0
Final volume in ml made with water		100	100	100	100	200	200	200

[a]Tributyrin agar well method.

Table A2. List of Chemicals Used in This Study

Chemical	Reference number	Company
Acetone	2240	Mallinckrodt
Agar	0410-01	Difco
Ammonium chloride	3384	Mallinckrodt
Ammonium hydroxyde	3256	Mallinckrodt
Ammonium oxalate	0746	Baker
Ammonium sulfate	3512	Mallinckrodt
Ascorbic acid	1852	Mallinckrodt
Beef Extract	0126-01	Difco
Butyl Alcohol (normal)	2990	Mallinckrodt
Calcium chloride	1-1332	Baker
Casein (Hammerstein quality)		Nutritional Biochemicals Co.
Chloroform	4440	Mallinckrodt
Citric acid	0627	Mallinckrodt
Crystal violet	C581	Fisher
Dextrose	4912	Mallinckrodt
Ethanol	USP	AAPER Alcohol and Chemical Co.
Gelatin	2124	Baker
Glycine	NDC 12894	U.S. Biochemical Co.
Gum arabic	G9752	Sigma
Hydrochloric acid	9535	Baker
Iodine	1008	Mallinckrodt
Lactose	L3625	Sigma
Phenolphtalein	6600	Mallinckrodt
Phenol red	P391	Mallinckrodt
Potassium acid phtalate	6704	Mallinckrodt
Potassium hydroxyde	6984	Mallinckrodt
Potassium iodide	3164	Baker
Potassium dibasic phosphate (anhydrous)	7092	Mallinckrodt

Table A2. (continued)

Potassium monobasic phosphate	7100	Mallinckrodt
Safranin O	SX20	Matheson, Colleman and Bell
Silicilic acid	2847	Mallinckrodt
Sodium acetate	7364	Baker
Sodium bicarbonate	S233	Fisher
Sodium chloride	7581	Mallinckrodt
Sodium desoxycholate	0248-13	Difco
Sodium hydroxyde	7708	Mallinckrodt
Sodium taurocholate	S07270	Pfaltz & Bauer
Sucrose	8360	Mallinckrodt
Sulfuric acid	2876	Mallinckrodt
Tributyrin	T-4637	Fisher
Tris (hydroxymethyl)aminomethane	907438	Schwartz/Mann
Tryptone	0123-01	Difco
Yeast Extract	0127-01	Difco