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PRODUCTION AND CHARACTERIZATION
OF
PENICILLIUM CASEICOLUM LIPASE

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PRODUCTION AND CHARACTERIZATION OF *PENICILLIUM CASEICOLUM* LIPASE

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ABSTRACT

PRODUCTION AND CHARACTERIZATION
OF PENICILLIUM CASEICOLUM LIPASE

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Penicillium caseicolum was grown in five fungal media. As compared to other media, mycological broth and Czapek-Dox broth were better for growth and lipase production. Five strains of Penicillium caseicolum were assayed for their lipase activity. All strains showed various degrees of lipase production. Strain B_5 displayed highest lipase production.

The nutrient requirement and influence of other environmental factors on growth and lipase production by Penicillium caseicolum C_1 were studied. Under submerged condition with agitation both growth and lipase production were higher than in stationary state. Soytone was an excellent source of nitrogen. Dextrose was found to be the best source of carbon in the growth medium. Certain minerals added to the growth medium inhibited the cell growth and lipase production to various degrees. Corn oil stimulated the growth and lipase production more than the other oils tested. The optimum conditions observed for growth and lipase production in the fermentor were 25°C, pH 7.0 and agitation at rate of 500 rpm.

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Lipase activity was assayed by pH-stat and silica gel chromatography. The optimum pH and temperature for the enzyme activity were 9.0 and 35°C, respectively. Lipase activity was either inhibited or stimulated by the addition of sodium taurocholate, sodium desoxycholate and calcium chloride, depending on the concentration of salts and type of substrate. The enzyme was stable at -26.6°C and -15°C for one month and for 3 days at 0° and 4°C. Most lipase activity was lost upon pasteurization and autoclaving treatments. The lipase lost its activity completely upon boiling for 6 minutes. The Z_D value was 15.8°C. With simple triglycerides as substrates, the enzyme displayed high specificity toward short chain fatty acids, especially butyric acid. The rate of hydrolysis of tributyrin was 4 times faster than triolein. Lipase activity toward natural lipids was highest with corn oil and sunflower oil. The enzyme hydrolyzed corn oil 1.5 times faster than butter oil, indicating a specificity of the enzyme for polyunsaturated fatty acid. Gas-liquid chromatographic analysis of lipolyzed butter oil showed butyric acid was the major free fatty acid released.

DEDICATION

TO MY WIFE RABAB

AND MY SONS

SINAN, GHAZWAN, AND FRAS

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INTRODUCTION

Proteins, carbohydrates and lipids are the main sources of flavor precursors in foods. Minor precursors such as polyphenols, nucleotides and pigments also partly contribute to the flavor development.

A large number of foods contain significant amounts of lipids susceptible to hydrolysis by lipolytic enzyme(s). The hydrolytic products contribute to both desirable and undesirable flavors in many foods, especially in fermented dairy products.

Lipases are widely distributed in nature. They are produced from different sources. Lipases are present in foods as inherent enzymes or specific enzymes added to the foods. Also microbial enzymes arise from contaminating organisms or microorganisms added to the foods (Shahani et al., 1976).

A number of microorganisms are known to produce lipolytic enzymes. Lipolysis, a major biochemical change occurring during ripening of cheese, is essential for the generation of proper flavor. Research in cheese flavor has shown that molds contribute significantly to the flavor of Roquefort, Stilton, Blue, Brie and Camembert cheese. Penicillium camemberti and Penicillium caseicolum (or Penicillium candidum) are used in Camembert and Brie cheese

production. The white mold grows on the surface of cheese and plays a significant role in the production of typical cheese flavor. Penicillium caseicolum produces an extracellular lipase which degrades milk fat to produce an array of flavor compounds of Camembert and Brie cheese. Limited information is available on production and characterization of the lipase of Penicillium caseicolum. Therefore, this investigation was conducted to study the growth of Penicillium caseicolum in relation to production and activity of the lipase. Effect of the addition of various sources of nitrogen, carbohydrates, minerals and oils, to the growth medium was studied. In addition, certain factors influencing the activity of the lipase were studied with an overall view to help understand the role of Penicillium caseicolum in the production of Camembert cheese flavor. It is also a step towards using lipase of Penicillium caseicolum for commercial uses.

LITERATURE REVIEW

Microorganisms are becoming main sources for a wide variety of industrial enzyme production because of their technical and economical advantages over the animal and plant sources. Selection of suitable strains and determination of optimum conditions make it possible to achieve high yield of desirable enzymes.

Microbial Characterization of *Penicillium caseicolum*

Penicillium camemberti Thom and *Penicillium caseicolum* Bainier belong to asymmetrica section of Penicillium genus and sub-section Lanata (Rapper and Thom, 1949). *Penicillium candidum* and *Penicillium album* correspond to *Penicillium caseicolum* and *Penicillium camemberti*, respectively (Raper and Thom, 1949; Samson *et al.*, 1977; Moreau, 1979). The colonies may possess pale green color in case of *Penicillium camemberti* and white color in case of *Penicillium caseicolum*. These two species differ slightly in their physiological and biochemical characteristics. These differences are utilized in the manufacture of Camembert cheese.

Samson et al. (1977) considered Penicillium camemberti, (Penicillium album) and Penicillium caseicolum (Penicillium candidum) as synonymous with the same taxonomy. Their colonies on Czapek-Dox agar grow slowly, attaining a diameter of 2-2.5 cm within two weeks at 25°C. A raised floccose aerial mycelium usually up to 1 cm high is observed. The mycelium remains white. Sometimes it changes to yellowish, grey-greenish, and in rare cases to pinkish color. Conidiogenous structure generally arises from submerged hyphae and occasionally from aerial hyphae. Conidiophore stipes are generally rough-walled and rarely smooth-walled. The conidiophore is up to 500 µm long, and 2.5-4 µm wide. Metulae are 8-14x2.5-3.0 µm, giving rise to 3-6 Phialides. It has a flask shape with a short neck, 10-13x 2.2-2.5 µm. Conidia are in tangled chains, globose to subglobose or broadly elliposidal, hyaline or slightly greenish 4.0-5.0x 3.0-4.5 µm.

Colonies on Czapek-Dox agar and malt agar are reportedly similar, but malt agar yields more conidiophores which display relatively more rough appearance. Most strains of Penicillium caseicolum remain white on media containing 3% sucrose concentration. Occasionally, they change to pale yellow color. Some strains of Penicillium caseicolum become yellow or even pinkish on Czapek-Dox agar. According to Tamime (1981) Penicillium camemberti produces pale-green colonies when grown on solid media. It shows abundant

conidial heads and conidiophores arising from aerial hyphae. Penicillium caseicolum conidia are uncolored. Both Penicillium camemberti and Penicillium caseicolum have moldy odor.

Relationship of Microbial Growth to Lipase Production

Production of lipase is influenced by the type of microorganism, composition of medium and condition of growth. Perlman (1971) stated that nutrient requirement for growth and enzyme production is related to growth cycle. The level of enzyme is in turn related to growth of the organism. Boing (1982) suggested that lipase production in synthetic media is highly dependent on the nutrients available as well as physical condition of growth medium.

Culture Medium

A wide variety of media for mold growth are available. Raper and Thom (1949) reported the following media for growth of Penicillium Sp.: Czapek's solution agar, malt extract agar, corn meal agar, wort or beer wort, bean agar and potato agar. Prescott and Dunn (1959) reported that molds in general showed good growth on malt medium and Czapek-Dox medium. Recommended liquid media are: Sabouraud Maltose broth, Sabouraud liquid medium, mycological broth and Czapek-Dox broth (Difco, 1971; Perlman, 1971; BBL, 1973;

Booth, 1971).

Booth (1971) stated that the selection of satisfactory medium for stimulating growth and sporulation of fungi should be found by actual experiment. Hankin and Anagnostakis (1975) suggested solid medium for production of lipolytic enzymes from fungi. Potato dextrose agar was used for growth and detection of lipase activity in species of Aspergillus, Cladosporium, Mucor and Trichodorma. El-Gendy and Marth (1980) used mycological agar for production of lipolytic activity by eighteen strains of Aspergillus flavus or Aspergillus parasiticus, one of Aspergillus ochraceus and 12 strains or species of Penicillium. Winifred et al. (1967) reported wheat bread crumbs as good medium for lipase production by Penicillium camemberti. Belloc et al. (1975) suggested that fermentation medium for growth and lipase production of Penicillium camemberti should contain sources of carbon, nitrogen, minerals and optional growth factors. The suggested medium consisted of peptone, 1.64%; glucose monohydrate, 1.09%; soya oil; 0.55%; NaCl, 0.55% and agar, 0.22%. The other formula consisted of corn-steep liquor (50% solids), 2.22%; soya oil, 1.67%; ammonium sulphate, 0.22%; and solution of cobalt chloride hexahydrate (concentration 20 g/l), 0.12%. Kosikowski (1977) reported a procedure for cultivating spores of Penicillium camemberti for cheese making. Penicillium camemberti is grown on Czapek-Dox agar or broth

supplemented with a stimulant (1% yeast extract) at 10°C and 90% relative humidity for one month. The spore suspension grows on crackers before inoculation to the cheese.

Influence of Nitrogen Sources

Numerous protein or protein degradation products are added to the medium for growth and lipase production. Cutchins et al. (1952) found that lipase production of Pseudomonas fluorescens was inhibited by addition of casein hydrolyzate, but glycine stimulated lipase production more than a peptone. It was suggested that lipase production decreased as the structural complexity of nitrogen increased. Nashif and Nelson (1953a) found fairly good growth and highest lipase production in medium containing proteins digest or hydrolyzates as a source of nitrogen. Lawrence et al. (1967) suggested peptone as the most satisfactory source of nitrogen for growth and lipase production of Pseudomonas fragi and Micrococcus sp. Mates and Sudakevitz (1973) reported that peptone inhibited lipase production of Staphylococcus aureus, even though the growth was normal. On the contrary, peptone stimulated lipase production of some species of Pseudomonas. Alford and Pierce (1963) compared lipase production of Pseudomonas fragi in buffered sulfate-glucose medium containing peptone with the medium containing arginine, lysine, aspartic acid and

glutamic acid . Lipase production in the medium supplemented with the amino acids was 90% of the lipase production in the medium containing peptone.

In general, molds can utilize a wide variety of nitrogen sources. Imamura and Kataoka (1963) found addition of casein or peptone to the growth medium of Penicillium roqueforti showed greater lipase production than the medium containing inorganic sources of nitrogen (sodium nitrate or ammonium sulfate). Eitenmiller et al. (1970) reported maximum lipase and growth for Penicillium roqueforti in medium containing 0.5% casitone and 1% proflo broth as sources of nitrogen.

Liu et al. (1972) showed the addition of 5% corn steep liquor to the medium increased the growth and lipase production by Humicola Lanuginsoa. Peptone promoted the growth, but repressed the lipolytic productivity of this microorganism. Chander and Ranganathan (1975) found alanine, glycine, lysine and serine to be stimulatory for the growth and lipase production of Streptococcus faecalis, while aspartic acid , cystine, phenylalanine, proline and tyrosine were not essential for growth and lipase production. Chander et al. (1977, 1980a, 1981) found that peptone increased growth and lipase production of Penicillium chrysogenum, Aspergillus wentii and Rhizopus nigricans.

Influence of Carbon Sources

Raper and Thom (1949) stated substitution of glucose for sucrose in growth medium enhanced the growth of certain strains and species of Penicillium. Different sources of carbon and media for the growth and lipase production of different microorganisms are: trypticase, soy broth, nutrient broth, corn oil, soybean meal, wheat bran, 2.5% kerosene, 2-4% protein hydrolyzate:0.25% glucose, 7% polypeptone: 2% glucose, 4% soluble starch, 3% soybean meal, 2% trypticase:5% glucose, 4% rice bran:3% corn steep liquor, and 2% soluble starch:2% soybean meal (Fukumoto et al., 1963; Takahashi et al., 1963; Mencher and Alford, 1965; Motai et al., 1966; Nagaoka et al., 1969; Finkelstein et al., 1970; Tsuisaka et al., 1973; Yamaguchi et al., 1973; Mates and Sudakevitz, 1973; Jonsson and Snygg, 1974; Bennett et al., 1976).

Nashif and Nelson (1953a) found glucose to be better than lactate for growth and lipase production of Psuedomonas fragi. However, Alford and Pierce (1963) reported the omission of glucose from growth medium of Psuedomonas fragi had little effect on growth, but no lipase was produced.

Imumura and Kataoka (1963) reported that lactose, glucose or galactose decreased growth and lipase production of Penicillium roqueforti. Eitenmiller et al. (1970) reported no significant lipase production by Penicillium

roqueforti when 1% saccharose was replaced with 1% lactose in Czapek-Dox broth. Mates and Sudakevitz (1973) reported glucose, mannitol and glycerol inhibited lipase production, but salicin, inulin and raffinose did not inhibit or stimulate the lipase synthesis of Staphylococcus aureus. Also all these carbon sources did not affect the growth substantially. Akhtar et al. (1973) found replacing glucose with olive oil as a source of carbon increased the mycelia and lipase activity of Rhizopus arrhizus, Rhizopus delemer, Rhizopus nigricans, Mucor javanicus, Penicillium roqueforti and Geotrichum candidum. The extracellular lipase decreased in case of Rhizopus sp. and Penicillium roqueforti, but increased with Mucor javanicus and Geotrichum candidum. Shen et al. (1974) indicated glucose had inhibitory effect on lipase production by Eremothecium ashbyii in oil medium. On the other hand, some workers reported glucose as best source of carbon for some fungi. Chander et al. (1977) found maximum growth and lipase production of Penicillium chrysogenum in medium containing glucose, followed by maltose, mannitol, galactose, sucrose, lactose and fructose. In another report, Chander et al. (1980a) showed that Aspergillus wentii produced high growth and lipase in media containing glucose followed by mannitol, fructose, galactose, sucrose, lactose and maltose. Chander et al. (1981) reported glucose to be best carbon source for growth and lipase production of Rhizopus nigricans. Galactose, fructose, sucrose, lactose and maltose were less effective for growth and lipase

production of Rhizopus nigricans.

Weete and Weber (1980) stated glucose was the most important carbon source for the growth of fungi. Monosaccharides such as fructose, mannose and galactose supported growth in some species. Some fungi utilized maltose better than glucose for their growth. Sucrose and trehalose were found to be good sources of carbon for most species.

Influence of Minerals

Prescott and Dunn (1959) stated certain elements such as Zn, Fe, Cu, Mg, Mo and Ga to be important for mold growth. Bridson and Breecher (1970) reported that most bacteria require K^+, Mg^{+2}, Mn^{+2}, Fe^{+2}, Fe^{+3} and SO_4^{-2} for their growth.

Nashif and Nelson (1953) reported addition of NaCl to growth medium inhibited lipase production by Pseudomonas fragi. Nadkarni (1971) found addition of 0.01% $MgSO_4 \cdot 7H_2O$ or 0.02% KH_2PO_4 to growth medium effected optimum lipase production by Pseudomonas aeruginosa. Mates and Sudakevitz (1973) reported that increasing the concentration of NaCl from 0.1M to 1.2M in growth medium caused proportional inhibition of lipase production without affecting growth of Staphylococcus aureus. The addition of sodium taurocholate to the growth medium did not affect the bacterial growth but reduced lipase activity.

Chander et al. (1980a) reported that addition of calcium and sodium citrate to growth medium stimulated

lipase production 46% and 64%, respectively by Aspergillus wentii. Potassium citrate had no appreciable effect on lipase production. In another report, Chander et al. (1981) found increased Rhizopus nigricans lipase production by addition of 0.1% calcium citrate, more than sodium citrate and potassium citrate.

Influence of Other Culture Condition

Each microorganism required certain culture condition for the growth and lipase production, such as submerged culture, aeration, agitation, temperature, and pH. Submerged (shaker) culture techniques are widely used for growth of bacteria, molds, yeasts and actinomycetes. The submerged culture generally provides more uniform and better growth (Calam, 1969). In submerged culture necessary oxygen is supplied to the microorganisms to achieve optimum metabolic activity and cell biosynthesis (Perlman, 1971; Wang and Cooney, 1979).

Several workers produced microbial lipase by submerged culture. Yoshida et al. (1968) obtained lipase from Torulopsis ernobii in a fermentor with agitation at 500 rpm. Other microorganisms used for lipase production are: Pseudomonas aeruginosa, Geotrichum candidum, Mucor pusillus, Humicola lunuginosa, Saccharomycopsis lipolytica, Micrococcus caseolyticus, Bacillus licheniformis, and Staphylococcus

sp. (Somkuti et al., 1969; Nadkarni, 1971; Jonsson and Snygg, 1974; Tsujisaka et al., 1973). Eitenmiller et al. (1970) found growth of Penicillium roqueforti was 28% higher in submerged condition than in stationary condition. Nelson (1970) obtained maximum blue cheese flavor from Penicillium roqueforti by submerged culture. The flavor resulted from enzyme hydrolysis of milk fat. Akhtar et al. (1974) reported lipase production by Rhizopus sp. and Penicillium roqueforti at 200 rpm. They also investigated the enzyme products of Mucor sp. and Geotrichum candidum at 150 rpm. Iwai and Tsujisaka (1974) obtained lipase from Rhizopus delemar by submerged culturing at 110 rpm, 7 cm amplitude for 4 days. Iwai et al. (1975) reported submerged production of lipase from Penicillium cyclopium at 110 rpm, 7 cm amplitude for different periods of time. Chander et al. (1980a) produced lipase from Aspergillus wentii under submerged culture at 200 rpm for 3 days.

Influence of Lipids Addition

Several investigators studied the effect of addition of lipids on lipase production by various microorganisms. Nashif and Nelson (1953) found addition of small amounts of tricaprylin, caprylic acid or capric acid to vitamin-free casamino acids or peptone media caused a pronounced increase in lipase production of Pseudomonas fragi. Smith

and Alford (1966) reported addition of lard or sodium oleate to the growth medium had no effect on growth, but decreased the lipase production of Pseudomonas fragi. Also lipase production by Geotrichum candidum was inhibited by lard, sodium oleate and salts of other fatty acids. Khan et al. (1967) found that the addition of 1% olive, corn or butter oil to their growth medium enhanced lipase production of Achromobacter lipolyticum by 87, 69 or 25%, respectively. Ota et al. (1968) noted lipase production of Candida paralipolytica was increased by addition of castor oil to growth medium. Coconut oil was not equally effective in inducing the lipase production. It was suggested that linseed oil, soybean oil, rape seed oil, olive oil, and coconut oil acted as growth accelerators. Later Ota et al. (1968a) found that a combination of two kinds of lipids e.g. cholesterol and olive oil was obligatory for lipase production by Candida cylindracea. Torulopsis ernobii growth and lipase production were increased by addition fat, oil and triglycerides containing higher fatty acids such as palmitic and oleic to the growth media (Yoshida et al., 1968). Eitenmiller et al. (1970) reported that the addition of butter oil, corn oil or olive oil to the growth medium inhibited lipase production of Penicillium roqueforti. Iwai et al. (1973) found that lipase production of Geotrichum candidum increased with addition of higher fatty acids or oils to the growth medium. It was suggested that oleic and

linoleic acids played a significant role in increasing lipase production.

Jonsson and Snygg (1974) found that the addition of olive oil to growth medium decreased lipolytic activity of Saccharomycopsis lipolytica, Micrococcus caseolyticus, Bacillus licheniformis and Staphylococcus sp. Jonsson (1976) studied Saccharomycopsis lipolytica and Micrococcus caseolyticus grown in nutrient broth emulsions of olive oil, soybean oil and linseed oil (20% fat concentration). Neither growth nor the amount of lipase varied with the kind of fat.

Sharma and Chauhan (1977) stated that fungi and actinomycetes have strong capacity for utilizing fatty substances such as triglycerides of highly unsaturated dienoic or trienoic fatty acids either in vitro or in vivo by secreting extra-cellular lipases.

Chander et al. (1979) found the addition of short chain fatty acids such as C_3, C_4, C_6, and C_8 into growth medium of Streptococcus faecalis had stimulatory effect, whereas C_{12}, C_{14}, C_{16} and C_{18} had inhibitory effect on growth and lipase production. In another report Chander et al. (1980, 1981a) found the addition of olive oil, butter oil, tributyrin, tricaproin, tricaprylin and tripropionin had inhibitory effect on growth and lipase production of Penicillium chrysogenum and Rhizopus stolonifer. Lipase production of Pseudomonas fluorescens was delayed

by addition of olive oil, sunflower oil or soybean oil to the growth medium (Anderson, 1980). Demoraes (1981) found Streptococcus thermophilus lipase production was inhibited by the addition of butter oil (29%), milk (41%), casein (12%) and stimulated by soybean oil (39%), cream (27%) and corn oil (21%).

According to Nieman (1954) and Eitenmiller et al. (1970) the inhibitory effect of addition of oil to the growth medium may be due to the bacteriostatic effect. Maxcy and Chandan (1962) ascribed the inhibition in growth to surface tension lowering by fatty acids, thereby causing interference with metabolism of the microorganisms.

Influence of Cultivation Temperature

Most molds grow best at 15 to 25°C (Raper and Thom, 1949). Penicillium sp. grow well at room temperature (23-25°C). Optimum temperature for growth and lipase production for some microorganism is presented in Table 1. The temperature ranges from 15 to 45°C, depending on the microorganism.

Belloc et al. (1975) suggested 25°C as optimum temperature for growth and lipase production of Penicillium camemberti.

Table 1. Cultivation temperature of various microorganisms for lipase production.

Microorganism	Temperature °C	Reference
Pseudomonas fragi	15 or below	Nashif and Nelson (1953a)
Alcaligenes viscosus	21	Nashif and Nelson (1953c)
Flavobacterium sp.	21	Nashif and Nelson (1953c)
Pseudomonas aeruginosa	21	Nashif and Nelson (1953c)
Pseudomonas fluorescens	15	Nashif and Nelson (1953c)
Pseudomonas sp.	21	Nashif and Nelson (1953c)
Pseudomonas viscosa	21	Nashif and Nelson (1953c)
Pseudomonas synxantha	21	Nashif and Nelson (1953c)
Serratia marcescens	21	Nashif and Nelson (1953c)
Achromobacter lipolyticum	32	Nashif and Nelson (1953c)
Pseudomonas fluorescens	20	Alford and Elliot (1960)
Pseudomonas fragi	22	Lawrence (1967)
Achromobacter lipolyticum	21	Khan et al. (1967)
Staphytococcus aureus	35	Mates and Sudakevitz (1973)
Chromobacterium viscosum	26	Yamaguchi et al. (1973)
Bacillus licheniformis	20	Jonsson and Snygg (1974)

Table 1. (cont'd).

Micrococcus caseolyticus	20	Jonsson and Snygg (1974)
Streptococcus thermophilus	44	DeMoraes (1981)
Penicillium roqueforti	25	Immura and Kataoka (1963)
Penicillium roqueforti	27	Eitenmiller et al. (1970)
Penicillium roqueforti		
Mucor pusillus	35	Somkuit and Babel (1968)
Penicillium camemberti	30	Winifred et al. (1967)
Penicillium camemberti	25-28	Belloc et al. (1975)
Penicillium chrysogenum	30	Chander et al. (1977)
Aspergillus wentii	30	Chander et al. (1980a)
Rhizopus nigricans	30	Chander et al. (1981)
Humicola lanuginosa	45	Liu et al. (1972)
Saccharomycopsis lipolytica	20	Jonsson and Snygg (1974)
Eremothecium ashbyii	40	Shen et al. (1974)

Influence of pH

Several workers reported pH 6-7 as optimum for growth of most fungi (Koburger, 1970; Booth, 1971; Jarvis, 1973; Ladiges et al., 1974; Weete et al., 1974; Weete and Weber, 1980). In most studies, the optimum pH for lipase production has been conducted without constant pH, using the initial pH of the growth medium (Lawrence, 1967). The optimum pH for lipase production by certain microorganisms is presented in Table 2.

Sartory et al. (1927) found the optimum pH of growth for Penicillium caseicolum to be 6.5-7.0. Lamberet and Lenoir (1972) used pH 7.3-7.5 for the same microorganism. In this regard, Belloc et al. (1975) suggested pH 6.5-8.0 for growth and lipase production of Penicillium camemberti. Kunz and Singer (1976) reported the maximum growth of the same organism at neutral pH.

Evaluation of Growth

Methods commonly utilized for microbial growth evaluation are based on turbidimetry, wet weight, dry weight, cell packing, nitrogen and microscopic determination. Dry weight is widely used and provides data proportional to the mass (Mallette, 1969). In this regard, the mycelial organisms, such as fungi, post special challenges. Fungi grow slowly and change in composition as they age. At any time a part

Table 2. Optimum pH for Microbial Lipase Production.

Microorganism	pH	Reference
Pseudomonas fragi	6.5-7.5	Nashif and Nelson (1953)
Achromobacter lipolyticum	7.0	Khan *et al.* (1967)
Staphylococcus aureus	7.5-9.0	Vedehra and Harmon (1967)
Staphylococcus aureus	7.5-8.8	Mates and Sudakevitz (1973)
Penicillium roqueforti	8.0	Niki *et al.* (1966)
Penicillium roqueforti	5.5	Eitenmiller *et al.* (1970)
Penicillium chrysogenum	6.0	Chander *et al.* (1977)
Aspergillus wentii	6.0	Chander *et al.* (1980a)
Rhizopus nigricus	6.0	Chander *et al.* (1981)
Candida mycoderma	5.5	Hosono and Tokita (1970)
Debaryomyces klocekeri	7.0	
Humicola languinosa	7.0	Liu *et al.* (1972)
Eremothecium ashbyii	6.8	Shen *et al.* (1974)

of the culture is growing while other parts are aging and dying. The composition of mycelium is also changing continuously. The mycelium may store reserve substanced and fat, so that a weight increase occurs without formation of new cells. In general, fungal growth may be evaluated by drying mycelium to constant weight (Calam, 1969a). Shih and Marth (1974) determined the growth of Aspergillus parasiticus by drying at 50°C for 24 h. Chander et al. (1977) evaluated the growth of Penicillium chrysognum by drying mycelium at 100°C for 24 h. Moreira et al. (1979) used dry cell weight to measure the growth of Candida lipolytica and Trichoderma viride at 80°C overnight.

Occurrence and Nature of Microbial Lipase

Yeasts, bacteria and molds possess the ability to produce lipolytic enzymes during their growth in foods or in suitable media (Lawrence, 1965; Wills, 1965; Jensen, 1971; Desnuelle, 1972; Shahani, 1975).

Pollock (1962) classified microbial enzymes according to their location:

1) Cell bound enzyme, divided into a) surface bound enzyme attached to surface of cell; b) intracellular enzyme bound to cytoplasm.

2) Extracellular enzyme, produced by cells and secreted into the medium.

Stanier et al. (1976) divided the enzymes produced by cell into three categories:

1) Exoenzymes. Enzyme synthesized within the cell and excreted into the growth medium.

2) Periplasmic enzymes. Produced between the cell membrane and the outer layer of the wall (periplasmic space). They are found in some gram-negative bacteria.

3) Exocellular enzymes, which are situated external to the cell membrane to which they remain tightly bound.

Arkinson (1973) classified microbial lipases into intracellular and extracellular enzymes. This classification agrees with Lawrence (1967). He reported that extracellular lipase is found in cell-free medium in a culture where cells are in log phase of growth. This would minimize chances of cell lysis and consequent spillage of the intracellular enzymes in the medium.

The intracellular enzymes are located within the cell and are discernible only by cell disruption. The extracellular enzymes are present in the medium and are relatively more pure than intracellular enzymes (Aunstrup et al., 1979; Boing, 1982).

Most lactic acid bacteria have only intracellular enzymes (Chandan et al., 1969; Chander and Chebbi, 1973; Formisano et al., 1974). Molds produce intracellular lipase, as well as extracellular lipase (Fukumoto et al., 1963; Tomizuka et al., 1966; Somkuti et al., 1969; Eitenmiller et al.,

1970; Benzonana and Esposito, 1971; Liu et al., 1973; Tsujisaka et al., 1973; Akhtar et al., 1974; Iwai et al., 1975; Lamberet and Lenoir, 1972, 1976).

Characterization of Microbial Lipases

Assay Methods

A number of methods are available for determining lipase activity. It can be measured by disappearance of the substrate or by appearance of free fatty acids released.

Qualitative Methods

Dye methods. Victoria Blue, Spirit Blue, Nile Blue sulfate, Night Blue and other dyes have been used for detection of lipolytic activity of microorganisms. Toxic effects of these dyes on some microorganisms have been documented (Lawrence, 1967; Bours and Mosel, 1973).

Agar diffusion method. This method involves in spreading tributyrin agar emulsion over a standard area of a microscopic slide or petri dish, cutting a hole in the agar and inoculating with lipase. The appearance of a clear zone around the hole indicates lipolytic activity (Lawrence, 1967).

Double layer method. This method is used for screening lipolytic microorganisms. The organisms are grown on nutrient agar overlaid either with tributyrin agar or thin layer of milk fat saturated with Victoria Blue (Fryer et al., 1967).

In all the above methods, plates have to be incubated at 20-25^{o}C for 3 days when tributyrin is used as substrate and 4-7 days for other fats (American Public Health Association, 1976).

Quantitative Methods

Procedures based on surface tension, colorimetry, Warburg monometric technique, turbidometric methods, photometric methods, gas chromatography and radioactive tracer techniques are available but not widely used (Shahani, 1975; Webb et al., 1978).

The most widely used methods are titration methods, based on the titration of fatty acid released, by enzyme action on the substrate. Fatty acids extracted on silica gel column are titrated with standard alkali (Harper et al., 1956).

The other titration method employs a pH-stat. The enzyme is incubated with substrate at constant pH and temperature. The titration occurs mechanically from an automatic burette. Titer value depends on the amount of fatty acids released by the action of enzyme (San Clementi and Vadehra, 1967; Parry et al., 1965; Shahani, 1975).

The pH-stat method measures initial velocity of reaction. It is rapid, accurate and more sensitive than other methods.

Temperature Optimum for Lipase Activity

The temperature of incubation of the enzyme substrate mixture is important and apparently affects the specificity (Alford and Pierce, 1961). Temperature optimum for lipase activity are listed in Table 3. The optimum temperature varies from 30-60^{o}C for various microorganisms and their strains. However, some microbial lipases are capable of hydrolyzing lipids at lower temperature. According to Alford and Pierce (1961) Pseudomonas fragi, Staphylococcus aureus, Geotrichum candidum, Candida lipolytica, Penicillium roqueforti and an unidentified Penicillium sp showed considerable activity within 2-4 days at -7^{o}C and within a week at -18^{o}C.

Table 3. Temperature optimum of microbial lipases.

Microorganism	Temperature °C	References
Serratia marcescens	37	Hugo and Beveridge (1962)
Pseudomonas fragi	54	Mencher & Alford (1967), Lu et al. (1969)
Achromobacter lipolyticum	37	Khan et al. (1967)
Staphylococcus aureus	45	Vadehara and Harmon (1967)
Lactobacillus plantarum	37	Umemoto et al. (1968)
Lactobacillus casei	37	Umemoto et al. (1968)
Streptococcus diacetilactis	45	Umemoto et al. (1968)
Propionibacterium shermanii	47	Oterholm et al. (1970)
Microbacterium thermosphactum	35-37	Collins et al. (1971)
Chromobacterium viscosum	65-70	Sugiura et al. (1974)
Streptococcus faecalis	40	Chander et al. (1979a)
Streptococcus thermophilus	45	Demoraes (1981)
Aspergillus niger	25	Fukumoto et al. (1963)
Mucor pusillus	50	Somkuti et al. (1969)
Mucor javanicus	40	Saiki et al. (1969)
Penicillium roqueforti	37	Eitenmiller et al. (1970)
Geotrichum candidum	40	Tsujisaka et al. (1973)
Rhizopus delemar	30, 35, 40	Iwai and Tsujsaka (1974)
Penicillium camemberti	25	Belloc et al. (1975)
Aspergillus wentii	25	Chopra et al. (1980)
Candida cylindracea	47.5-57.5	Tomizuka (1966)

Table 3. (cont'd.).

Torpulopsis ernobii	45	Motai et al. (1966), Yoshida et al. (1968)
Candida mycoderma	35	Hosona and Tokita, 1970
Debaryomyces kloeckeri	30	Hosona and Tokita, 1970
Humicola lanuginosa	60	Liu et al. (1973)
Eremothecium ashbyii	40	Shen et al. (1974)

pH Optimum for Lipase Activity

According to Lawrence (1967) pH alters rate of lipolysis by changes in activity and stability of the enzyme, the velocity of enzyme-substrate combination and breakdown. In the case of an emulsion substrate the properties of substrate-aqueous phase interface may be affected by pH. Some workers (Nashif and Nelson, 1953; Rottem and Razin, 1964) suggested that optimum pH may depend upon the nature of substrate being hydrolyzed, the buffer and other conditions of the assay. Hugo and Beveridge (1962) have pointed that the term optimum pH can only be a relative one since the temperature is arbitrarily fixed and all the above factors also affect the optimal lipolysis temperature. pH optimum for various microbial lipase activity are listed in Table 4. Weete et al. (1974) stated that fungal lipases appear to have optimum pH around 8.0. A wide range in both acid and alkali pH is reported. Factors affecting pH optimum are: organisms, intra or extracellular lipase, degree of purity, substrate, temperature and the other conditions of the assay.

Table 4. pH optimum of various microbial lipases.

Microorganism	pH	Reference
Achromobacter lipolyticum (intracellular)	9.0	Shahani et al. (1964)
Achromobacter lipolyticum	7.0	Khan et al. (1967)
Staphylococcus aureus (purified)	8.3	Vadehra and Harmon (1967a)
Micrococcus freudenrichii	8.0-8.5	Lawrence et al. (1967)
Pseudomonas fragi	8.0-8.5	Mencher and Alford (1976), Lawrence et al., (1967) and Lu et al., (1969)
Lactobacillus casei	7.0	Umemoto et al. (1968)
Lactobacillus plentarum	7.0-8.0	Umemoto et al. (1968)
Lactobacillus helveticus	7.0	Umemoto et al. (1968)
Streptococcus diacetilactis	6.5	Umemoto et al. (1968)
Propionibacterium shermanii	7.2	Otterholm et al. (1970)
Pseudomonas aeroginosa (purified)	8.0	Nadkarni (1971a)
Microbactrum thermos-phactum	7.1-7.3	Collins et al. (1971)
Corynebactrium acnes (partially purified)	7.5-9.0	Hassing (1971)
Lactobacillus brevis	6.5	Chander et al. (1973)
Micrococcus caseolyticus	9.5	Jonsson and Snygg (1974)
Bacillus licheniformis	8.5	Jonsson and Snygg (1974)
Staphylococcus sp	8.5	Jonsson and Snygg (1974)
Chromobacterium viscosum	7.0	Sugiura et al. (1974)
Streptococcus faecalis	7.5	Chander et al. (1979a)
Streptococcus thermophilus	9.0	Demoraes (1981)

Table 4. (cont'd).

Aspergillus niger	5.6	Fukumoto et al. (1963)
Penicillium crustosum	9.0	Oi et al. (1967)
Mucor javanicus	7.0	Saiki et al. (1969)
Mucor pusillus	5.0-5.5	Somkuti et al. (1969)
Penicillium roqueforti	6.5-6.8	Fodor and Chari (1949)
Penicillium roqueforti	5.5	Shipe (1951)
Penicillium roqueforti	8.0	Eitenmiller et al. (1970)
Rhizopus arrhizus	7.0	Semeriva et al. (1969)
Geotrichum candidum (crystallized)	5.6-7.0	Tsujisaka (1973)
Mucor lipolyticus	8 and 9	Nagaoka and Yamada (1973)
Rhizopus delemar	5.6-6.0	Iwai and Tsujisaka (1974)
Penicillium roqueforti (intracellular)	7.0	Kornacki et al. (1979)
Aspergillus wentii	5.5	Chopra et al. (1980)
Torulopsis ernobii	6.5	Motai et al. (1966) and Yoshida et al. (1968)
Candida cylindracea	5.2	Tomizuka et al. (1966)
Candida cylindracea (purified)	8.0	Ota et al. (1970, 1972)
Candida mycoderma	4.5	Hosono and Tokita (1970)
Debaryomyces kloeckeri	4.5	Hosono and Tokita (1970)
Humicola lanuginosa	8.0	Liu et al. (1973)
Saccharomycopsis lipolytica	9.5	Jonsson and Snygg (1974)
Eremothecium ashbyii	8.0	Shen et al. (1974)

Effect of Addition of Bile Salts and $CaCl_2$ on Lipase Activity

Microbial lipase activity may be stimulated by the addition of bile salt. According to Wills (1965), the role of bile salts appears to involve exact alignment of enzyme molecules in the interfacial layer. Benzonana and Desnuelle (1968) stated that in case of pancreatic lipase, bile salts are not true activators, but enable the reactions to proceed in a zero order rate. The action of bile salt on lipase activity depends on the degree of enzyme purification, temperature, pH, concentration of bile salt and the nature of the microbial lipase.

Several workers reported a stimulatory effect of the addition of bile salts to the reaction mixture (Nashif and Nelson, 1953; Shahani et al., 1964; Oi et al., 1969; Ota and Yamada, 1966; Nagaoka and Yamada, 1973; Yamaguchi et al., 1973; Sugiura et al., 1974; Wang, 1980).

On the other hand, the addition of bile salt had an inhibitory effect as reported by other workers (Fodor and Chari, 1949; Saiki et al., 1969; Oterholm et al., 1970; Finkelstein, 1970; Sugiura et al., 1974). Other reports indicate no effect of bile salt on the activity of microbial lipases (Nadkarni, 1971a; Yamaguchi et al., 1973; Sugiura et al., 1974). Bashkatova et al. (1976) found the addition of bile salt to the growth medium or directly to the reaction mixture of lipase of Pseudomonas fluorescens,

Serratia marcescens, Mycobacterium mucosum and Escherihia coli. Bile salts act in a complicated and probably indirect way depending on chemical composition of the salt. The bile salt affected both exo- and endo-lipases of these organisms. Chander et al. (1979a) reported that 0.2% bile salts stimulated lipase activity of Streptococcus faecalis.

Calcium activation of lipase has been reported by many investigators. According to Shipe (1951), addition of 10 mg of $CaCl_2$ to 12 ml of substrate increased the lipase activity of Penicillium roqueforti and Aspergillus niger. Iwai et al. (1964) obtained similar results for Aspergillus niger lipase by the addition of $CaCl_2$. Oi et al. (1969) reported the stimulatory effect of the addition of $CaCl_2$ and ferric sulfate to substrate of Rhizopus lipase. Extracellular lipase of Achromobacter lipolyticum was stimulated by the addition of $CaCl_2$, $MgCl_2$, Na_2SO_4, $MgSO_4$ and NaCl (Khan et al., 1967). Nadkarni (1971a) reported Ca ions stimulated lipase activity of Pseudomonas aeruginosa.

Inhibitory effect of calcium chloride on lipase activity has also been reported by Oterholm (1970). Other reports showed no influence of addition of calcium chloride on lipase activity (Rotten and Razin, 1964; Eitenmiller et al., 1970).

Stimulatory effect of Ca^{++} results from removal of fatty acids formed during the hydrolysis as insoluble calcium soaps. The calcium soap activates the hydrolysis by changing the interfacial substrate-water relationship

to one more favorable for enzyme action (Iwai, 1964). Also, Ca^{++} may inhibit the resynthesis of ester linkages, which would effectively shift the reaction in the direction of hydrolysis (Lawrence, 1967).

Thermostability of Microbial Lipases

Lipolytic enzymes and lipolytic organisms contained in foods may survive the food processing treatments. On storage of foods, they may enhance or cause deterioration of lipids (Alford et al., 1971; Jonsson and Snygg, 1974). The thermostability of microbial lipase varies considerably among organisms. Nashif and Nelson (1953b) found lipase from Pseudomonas fragi degraded fat at -10°C. Tomizuka et al. (1966) reported lipase of Candida cylinderaca to be stable at 15°C. It lost 5% of its activity in frozen state for one month.

Motai et al. (1966) showed that lipase from Torulopsis sp was stable at pH 3-8 at 37°C for 1 h and at pH 5.0 for 10 min. A rapid inactivation was observed above 70°C. Oi et al. (1969) studied thermal inactivation of some Rhizopus fungal lipases used in milk flavoring. The lipase did not lose activity at 47°C for 15 min. Liu et al. (1972) found lipase of Humicola lanuginosa to retain 100% of its activity at 60°C for 2 h. Only 35% lipase activity remained at 70°C after 20 min. Geotrichum candidum

lipase was stable when stored below 55°C for 15 min (Tsujisaka et al., 1973). The purified Mucor lipase was stable below 45°C but was inactivated at 60°C after 1 h. Purified lipase of Humicola lanuginosa was stable under 60°C and retained 55% of activity at 70°C after 20 min (Liu et al., 1973).

Sugiura et al. (1974) reported purified lipase A of Chromobacterium viscosum var paralipolyticum less stable than the crude enzyme and was inactivated rapidly above 50°C. Iwai and Tsujisaka (1974) reported crude lipase of Rhizopus delemar to be stable at various temperatures at pH 5.6. Purified enzyme B and C of Rhizopus delemar were stable below 45°C while A was stable even at 60°C. Iwai et al. (1975) found two lipases of Penicillium cyclopium were stable at 30°C for 15 min. Jonsson and Snygg (1976) determined lipolytic activity of Pseudomonas fluorescens at various temperatures. The storage stability at 37°C decreased with increasing the age of the culture. The activity disappeared after 10 days. Chander et al. (1979) reported purified lipase of Streptococcus faecalis was stable for 1 month at -18°C and completely inactivated at 90°C after 10 min.

Adams and Brawley (1981) found an extra-cellular lipase produced by Pseudomonas sp MC50 to be extremely heat resistant at 100-150° in water or emulsion. Lamberet and Lenoir (1976) found Penicillium caseicolum lipase was

stable at 30°C within pH 7.0-8.5. The stability was low at 37°C and pH 8.0. In general, thermostability of a lipase varies with the type of lipase, or microorganism, its strain, degree of enzyme purification, substrate and pH.

Table 5 presents a summary of thermal stability of various microbial lipases. It appears that fungal lipases are relatively stable when compared to pancreatic lipase (Weete et al., 1974).

Table 5. Thermalstability of various microbial lipases.

Microorganism	Loss of activity %	Temperature C	Time	Reference
Pseudomonas fragi	50	71.5	30 min	Nashif and Nelson (1953a)
Pseudomonas fragi	27.6	63.0	30 min	Mencher and Alford (1967) and Lu and Liska (1969)
Achromobacter lipolyticum (extracellular)	47	71.5	30 min	Khan et al. (1967)
Achromobacter lipolyticum	100	99	40 min	Khan et al. (1967)
Staphylococcus aureus	80	65	30 min	Vedehra and Harmon (1967a)
Propionibacterium shermanii	75	65	30 min	Oterholm et al. (1970)
Alcaligenis viscolactis	100	90-92	10 sec	Diessern and Stadhouders (1971)
Lactobacillus brevis	100	30	30 min	Chander et al. (1973)
Lactobacillus brevis	100	71.7	16 sec	Chander et al. (1973)
Aspergillus niger	50	60	15 min	Fukumoto et al. (1963)
Rhizopus delemar C	60	55	15 min	Tsujisaka et al. (1967)
Penicillium crustosum I and II	80	60	15 min	Oi et al. (1967)
Mucor javanicus	60	60	10 min	Saiki et al. (1969)

Table 5. (cont'd.).

Rhizopus arrhizus	0	30	2 h	Semeriva *et al.* (1969)
Penicillium roqueforti	65	37	60 min	Eitenmiller *et al.* (1970)
Penicillium roqueforti	90	40	60 min	Eitenmiller *et al.* (1970)
Penicillium roqueforti	100	50	10 min	Eitenmiller *et al.* (1970)
Candida cylindracea I	82	65	30 min	Tomizuka *et al.* (1966)
Candida cylindracea II	64	65	30 min	Tomizuka *et al.* (1966)
Torulopsis ernobii	0	65	10 min	Motai *et al.* (1966) and Yoshida *et al.* (1968)
Candida paralipolytica (purified)	45	45	20 min	Ota *et al.* (1970, 1972)

Action of Microbial Lipases on Natural and Synthetic Lipids

According to Alford et al. (1964) Penicillium roqueforti lipase preferentially hydrolyzed short chain fatty acids from butter fat. Eitenmiller et al. (1970) found the same organism to be more specific for tributyrin. The rate of hydrolysis in decreasing order was: tributyrin, tricaprin, tripropionin and triolein.

Hassing (1971) observed that lipase activity of Corynebacterium acnes was highest against tributyrin. Yamaguchi et al. (1973) studied the action of Chromobacterium viscosum on a variety of natural fats and oils. The lipase was more active against lard and butter than against olive oil. Chander et al. (1973) found lipase activity of Lactobacillus brevis to be greater for simple triglycerides. Tripropionin was attacked more easily than tricaprion, tricaprylin and more readily than butter oil and coconut oil. Sugiura et al. (1974) used different substrates for purified lipase A of Chromobacterium viscosum var paralipolyticum. The lipase was more active toward water insoluble triglyceride such as tributyrin or tripropionin, but exhibited a weak activity toward water soluble substrate such as triacetin or tweens. Iwai et al. (1975) found the activity of both A and B lipases of Penicillium cyclopium was high against vegetable oils such

as olive oil and popy oils. The action of lipase on tung oil and linseed oil was remarkably low.

Chander et al. (1979a) purified Streptococcus faecalis lipase. The enzyme hydrolyzed tributyrin > tricaprion > tricaprylin > triolein. The rate of hydrolysis of natural oils was as follows: butter oil > olive oil > linseed oil > coconut oil. On the other hand, Chopra et al. (1980) reported lipase of Aspergillus wentii preferentially hydrolyzed tricaprylin as compared to tricaproin, tripropionin and tributyrin. They reported the hydrolysis of butter oil was greater than olive oil, followed by coconut oil, mustard oil, and cottonseed oil.

Nature and Specificity of Lipolytic Enzymes

Lipolytic enzymes, such as lipases and esterases are involved in degradation and metabolism of fat. Shahani (1975) described the lipolytic enzyme as a hydrolase, catalyzing hydrolysis of the carboxylic acid ester bonds. Desnuelle (1972) differentiated lipases from esterases on the basis of physical state of the substrate. A lipase attacks substrate molecules in emulsion or insoluble form. An esterase attacks the substrate present in true aqueous solution. Wills (1965) and Jensen (1971) also defined lipase as an enzyme hydrolyzing the ester bond in emulsified glycerides at an oil-water interface in an insoluble

or heterogeneous system.

According to the International Union of Biochemistry (1961) lipase is a common name for "glycerol ester hydrolase" E.C. 3.1.1.3 that catalyses the deacylation of acylyglycerides and phospholipids.

The action of lipase on triglycerides is illustrated in Figure 1.

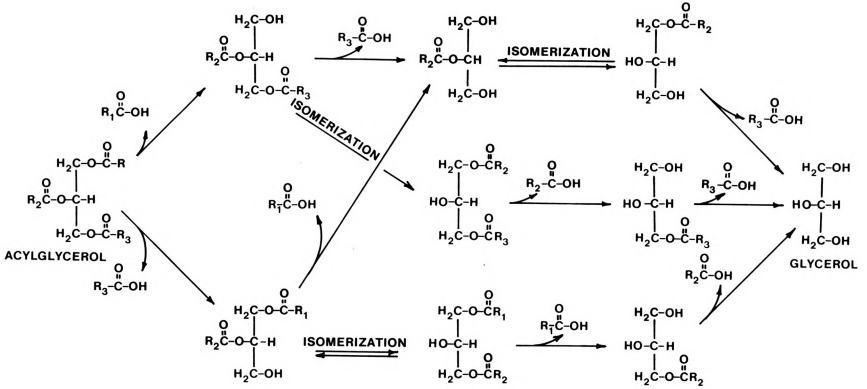


Figure 1 Lipase degredation of triglycerides
Source: Weete, J.D.; Webber, D.J. and Hess, W.M. (1974)

Lipases differ in relation to substrate, positional and fatty acid specificity (Desnuelle and Savary, 1963; Brockerhoff and Jensen, 1974; Shahani, 1975; Weete et al., 1974). The hydrolysis of monoglycerides to glycerol and fatty acids is generally very slow due to the isomerization of 2 monoglycerides to position 1 and 3 (Borgstrom and Ory, 1970).

Shipe (1951) and Eitenmiller et al. (1970) reported that lipase of Penicillium roqueforti was specific for short chain fatty acids.

Alford et al. (1964) reported that most lipases from fungi preferentially cleave fatty acids from C-1 position of triglycerides. Aspergillus flavus showed no positional specificity. Geotrichum candidum had specificity for Δ^9 unsaturation regardless of its position on triglyceride molecule. Jensen (1974) investigated the specificity of a purified extracellular lipase of Geotrichum candidum had no absolute specificity for fatty acids containing cis 9 or Cis 9,12 unsaturation. The enzyme hydrolyzed both the fatty acids regardless of position in triglycerides. Rhizopus arrhizus lipase has specificity for position C-1 and C-3 on triglycerides. Under certain conditions the C-2 acyl group migrates to position 1 and 3 (Weete et al., 1974; Semeriva et al., 1967). The extracellular lipase of Mucor pusillus shows maximal activity toward C_{12} fatty acid (Somkuti and Babel, 1968). Mucor javanicus preferentially cleavaged the ester bonds of C-1 and C-3 position

of triglycerides (Weete and Weber, 1980).

Lactic acid bacteria show preference for short chain fatty acids (Fryer et al., 1967a; Umemoto et al., 1968; Oterholm et al., 1970; Dovat et al., 1970; Chander et al., 1973; Formisano et al., 1974; Chander et al., 1979a). Also these bacteria are reported to hydrolyze complex and neutral lipids with higher fatty acids (Umemoto et al., 1968; Angeles and Marth, 1971; Chander et al., 1979).

Role of Mold in Cheese Flavor

During cheese ripening, a number of chemical reactions proceed. Many of the reactions are mediated by microbial and native milk enzymes (Scott, 1972a; Dwivedi and Shahani, 1973). Proteins, fats and carbohydrates are degraded to varying degrees to yield a complex mixture of compounds, which contribute to cheese flavor (Pack et al., 1968). Scott (1972) stated that ripening cheese is essentially an enzymatic process, at death, of bacteria and molds assist in the generation of the final flavor.

In the mold-ripened cheeses, Roquefort, Gorgonzola, Stilton, and blue cheese, the Penicillium roqueforti enzymes play important roles in flavor development. The blue-green mold produces substantial amount of lipase which leads to accumulation of free fatty acids, mainly caprylic, capric and caproic acids which in turn are involved in the formation of

flavoring compounds (methyl ketones) (Margalith and Schwartz, 1970). The methyl ketones, especially 2-heptanone and 2-nonanone are generally considered as the key flavor components of blue cheese (Day, 1976). Hawke (1966) stated that the formation and metabolism of methyl ketones by fungi in mold ripened cheeses consist of four main enzymatic mechanisms:

1) Liberation of free fatty acids from the triglycerides of milk fat by lipases.
2) Oxidation of the free fatty acids to β-keto acids.
3) Decarboxylation of β-keto acids to methyl ketone.
4) Reduction of methyl ketone to secondary alcohol.

Some workers report Penicillium roqueforti spores as well as mycelium are capable of producing methyl ketones from fatty acids (Gehrig and Knight, 1958; Lawrence, 1968; Dwivedi and Kinsella, 1974).

Some investigators studied the lipase of Penicillium caseicolum. Lamberet and Lenoir (1976) found the optimum pH of the crude lipase to be 8.5 at 30°C. The purified lipase had optimum pH of 9.0-9.6 and optimum temperature of 35°C (Lamberet and Lenoir, 1976a).

Moinas et al. (1975) reported contribution of certain minor compounds in Camembert cheese flavor. Such compounds are aromatic, hydrocarbons and ketones, unsaturated aliphatic ketones, aromatic esters, an aldehyde containing sulfur and a nitrite. Proks and Cingrosova (1962) showed that

Penicillium caseicolum lipase attacked preferentially the lower chain saturated fatty acids. Little activity towards the unsaturated acids was observed.

According to Kornacki et al. (1979) lipase of Penicillium roqueforti and Penicillium candidum released fatty acids with chain length of C_4 - C_{10} at both pH 6.6 and 8.6. The amount of C_4 and C_6 acids liberated were up to 10 times as high as their content in milk fat which was used as the substrate. Adda et al. (1978) reported Penicillium caseicolum plays a major role in Camembert cheese flavor. The free fatty acids present in large quantity contribute to the overall cheese flavor and serve as a substrate for the formation of methyl ketone and 2-alkanols. It was found that oct-1-en-3-ol was specifically involved in the pleasant mushroom flavor in Camembert cheese (Moinas et al., 1973; Dumont et al., 1974; Groux and Moinas, 1974). Oct-1-en-3-ol was shown to be produced by several Penicillium sp by oxidation of unsaturated fatty acids. An atypical off-flavor has been observed in cheese with some strains of Penicillium caseicolum which produce large amount of oct-1-en-3-ol. The proteolytic activity of Penicillium camemberti or Penicillium caseicolum is also fundamental for cheese ripening and the typical flavor of Camembert cheese. The proteolytic activity of Penicillium caseicolum was reported by several workers (Proks and Cingrosova, 1962; Lenoir and Choisy, 1971; Gripon et al., 1977).

Soltys et al. (1973) found that, during Camembert cheese ripening, there was a decrease in the level of casein to 50% of the original level. Simultaneously, there is an increase in nonprotein nitrogen to 25% of total nitrogen, ammonium nitrogen to 20% of soluble nitrogen, and amino nitrogen to 10% of soluble nitrogen. Also triglycerides decreased from 90% to 70% and free fatty acids increased from 1 to 10%, with very little increase in mono- and diglycerides.

MATERIALS AND METHODS

Four strains of Penicillium caseicolum were procured from G. Roger Laboratories (B.P. 20, 77260 La Ferte Sous Jouarre, France), and one strain was a gift from Centro Sperimeatale Del Latte (Via Salasco 4, Italy). Roger Laboratories recommend the mold growth on solid media containing sugar and peptone at 23°C and 70% relative humidity. The specification data show that the first germination appears within 6-9 h. After 11 h of cultivation, 70-95% of spores germinate. The mold grows at pH 4-8. The time for growth varies from one strain to another. Penicillium caseicolum is a halophilic organism capable of tolerating salt concentration used in cheese making. The main characteristics of different strains of Penicillium caseicolum are listed in Table 6.

Spore Count

The spores of Penicillium caseicolum were counted by using improved Neubauer Haemocytometer (Thomas, A.H. Co. 1981).

Penicillium caseicolum was grown on Czapek-Dox agar slant for one week at 25 ± 1°C. The spores were scraped from the slant under aseptic conditions using sterilized 0.1 M phosphate buffer, pH 7.0. The spores were suspended

Table 6. Characteristics of different Penicillium caseicolum strains*.

Mold strain	Main characteristics
Penicillium caseicolum C_1 and C_2	Produces aerial and dense mycelia. Gives medium mycelia layer on cheese. The growth is more difficult on curd poor or low in lactose content, leading to an earlier sporulation.
Penicillium caseicolum B_5	Requires low energy. Produces dense layer of mold and regular growth in ripening room. Grows well in poor or low lactose curd.
Penicillium caseicolum K_5	A rustic strain that produces dense mycelia. Growth is not influenced by composition of media. It should be mixed with other strains in cheese making.

*G. Roger Laboratories Manual.

in 100 ml of the phosphate buffer. One drop of the suspension was placed on Haemocytometer. The spores were counted under a microscope and the total count calculated as follows:

1. The average spore count of 10 small squares multiplied by 16 x 25, to get the count in large square.
2. Large square count multiplied by 10,000 to get the counts in 1 ml.

Fungal Maintenance and Inoculation

The commercial lyophilized spores of Penicillium caseicolum were suspended in 10 ml of sterilized distilled water and transferred to sterilized Czapek-Dox agar slant. The slant was incubated at 25 ± 1°C for 1 wk. The culture was transferred daily to new slants. In general, Penicillium caseicolum culture grown for 1 wk was used in all stages of this investigation. The growth from a slant was suspended in 100 ml sterilized 0.1 M phosphate buffer, pH 7.0. One percent of this suspension was inoculated in the broth media used in this study.

Growth Media

Five media commonly used for growth of fungi were investigated for growth studies. They were: Czapek-Dox broth, Sabouraud Maltose broth, Sabouraud Liquid medium, Maltose extract broth and mycological broth (Difco Lab). The composition of these media are listed in Table 7.

Table 7. Composition of growth medium (per liter).

Medium	Composition	pH
Czapek-Dox Broth	Saccharose Difco 30 g	
	Sodium Nitrate 3 g	
	Dipotassium phosphate 1 g	
	Magnesium sulfate 0.5 g	7.3
	Potassium chloride 0.5 g	
	Ferrous sulfate 0.01 g	
Sabouraud Maltose broth	Neopeptone 10 g	5.6
	Maltose Difco 10 g	
Sabouraud Liquid medium	Neopeptone 10 g	5.7
	Bacto-Dextrose 20 g	
Mycological broth	Bacto soytone 10 g	7.0
	Bacto Dextrose 40 g	
Malt extract broth	Special extract of malt by Difco	4.7

Source: Difco manual of dehydrated culture and media reagents (1972, Ninth edition, Difco Laboratories).

The media were sterilized at 121°C (15 psi) for 15 min. Spores and mycelia suspension of Penicillium caseicolum (5 ml) were inoculated in 500 ml media. The media were incubated at 23 ± 1°C under stationary conditions. The growth was harvested after 4, 7, 10 and 14 days of incubation. Dry cell weight were used to evaluate the growth of Penicillium caseicolum. Cell growth in Czapek-Dox broth and mycological broth was better than in other media. Therefore these media were used in subsequent work.

Influence of Stationary and Submerged (Shaking) Condition on Growth and Lipase Production of Penicillium caseicolum

This investigation was carried out using 100 ml each of Czapek-Dox broth and mycological broth. The media were sterilized at 121°C for 15 min (15 psi) and inoculated with 1% suspension of spores and mycelia of Penicillium caseicolum. The media were incubated under stationary as well as submerged (shaking) conditions at 25 ± 1°C. The flasks were shaken at 120 rpm (shaker Model G-25 New Brunswick Scientific Co., New Brunswick, NJ). The fungal growths were harvested at 4, 7, 10 and 14 day intervals. The lipase activity was determined in cell-free broth.

Influence of Oil Addition to the Growth Media of Penicillium caseicolum

Butter oil, corn oil and olive oil were chosen for this study. The investigation was carried out using 100 ml each

of Czapek-Dox broth and mycological broth containing 1% of the oil in 250 ml Erlenmeyer flasks. The media were sterilized at 121°C for 15 min under 15 psi. One percent suspension of Penicillium caseicolum spores and mycelia were inoculated in the media. Incubation under submerged (shaking) condition was at 25 ± 1°C (room temperature) and 120 rpm. The lipase activity in cell-free broth were determined at 4 and 7 days of culturing, also on daily basis.

Influence of Incorporating Soytone into Czapek-Dox Broth on Lipase Production by Penicillium caseicolum

Mycological broth contains soytone while the Czapek-Dox broth does not contain any organic N source. The experiment was designed to determine the effect of incorporating soytone and different oils into Czapek-Dox broth on lipase production. To 100 ml of Czapek-Dox broth 1% each of soytone and the oil was added in 250 ml Erlenmeyer flasks.

Growth conditions were similar to those described earlier.

Influence of Medium Composition on Growth and Lipase Production of Penicillium caseicolum

Mycological broth (composition: 4% dextrose, 1% soytone) was used as a basal medium for studying the influence of various components on growth and lipase production by Penicillium caseicolum. Corn oil (1%) was added to the

medium in all the studies, unless otherwise indicated.

Effect of Nitrogen Sources

In this phase of the study, soytone in mycological broth was replaced with equivalent concentration (1%) of casein, casamino acid, trypticase, proteose peptone, peptone, sodium caseinate, whey protein concentrate (29%) or whey protein concentrate (20%) as a source of nitrogen.

Effect of Carbon Sources

The dextrose was replaced with the same level of lactose, fructose, maltose, galactose and sucrose in the basal medium. Growth and harvesting procedures were the same as described earlier.

Influence of Minerals Addition to Growth Medium

An experiment was conducted to study if incorporation of selected minerals in the growth medium would influence growth and lipase production by the organism.

Various minerals were added to mycological broth medium supplemented with 1% corn oil. The minerals were: $NaNO_3$, 0.3%; K_2HPO_4-$7H_2O$, 0.1%; $MgSO_4$, 0.05%; KCl, 0.05%; $FeSO_4 7H_2O$, 0.001%; and $CaSO_4$, 0.01%. The sterilized media were inoculated with 1% suspension of the culture of Penicillium caseicolum under aseptic conditions. For growth, submerged (shaking) condition at 120 rpm and 25 ± 1°C was used. The media were harvested after 3 days for evaluation of growth and lipase production.

Influence of pH, Temperature and Agitation on Growth and Lipase Production of Penicillium caseicolum

The influence of these factors was studied by cultivation in a 7-liter bench-top fermentor (Microferm Fermentor, New Brunswick, NJ) equipped with agitation, aeration and automatic temperature control. The fermentor was connected to a constant pH controller (Virtis Digital pH controller, model 43 DPH CR) which was operated with a Virtis pH pump, model 43 PH-PE (Virtis Company, Gardiner, NY) and autoclavable pH electrode (Ingold Electrodes, Inc., Andover, MA). When the pH of the culture medium fell below the desired pH, the system delivered appropriate amount of 1 N NaOH to keep it at the desired pH. The culture vessel, air filter and tubings were autoclaved under 15 psi and at 121°C for 1 h. Three liters of mycological broth containing 4% dextrose, 1% soytone and 1% corn oil were prepared and placed in the culture vessel. The medium was sterilized in the culture vessel under 15 psi and at 121°C for 20 min, cooled to room temperature and inoculated aseptically with 1% suspension of spores and mycelia of Penicillium caseicolum.

1) Influence of pH was investigated at pH 4.7, 5.5, 6.5, 7.0, 7.5 and 8.5. The temperature was maintained at 25 ± 1°C, aeration at 200 cc/min and agitation at 120 rpm.

2) Influence of temperature. Experiment was carried out at 25°C and 30 ± 1°C. Other conditions of growth were: agitation 120 rpm, aeration 200 cc/min, and pH 7.0.

3) Influence of agitation of the medium. This study was conducted at constant pH 7.0, and at 25 ± 1°C. The aeration rate was 200 cc air/min. Agitation rates were 120, 200 and 500 rpm.

In all these investigations, the pH electrode was autoclaved at 15 psi and 121°C for 15 min. It was also chemically sterilized using lysol and alcohol before placing it in growth medium. The NaOH solution was also sterilized at 121°C/20 min. All other parts were chemically sterilized before their assembly to the fermentor.

The evaluation of growth and lipase production was carried out on daily basis for 5 days.

Measurement of Growth

The growth of Penicillium caseicolum was evaluated at all stages of this investigation by dry cell weight. The method was adopted from the procedures of Calam (1969) and Mallette (1969) to establish specific conditions to obtain constant weight of dry cells. Drying of the mycelial cells was done on a Whatman filter paper #5 which was itself dried at 100°C under vacuum (30 inch) for 5 h. A constant weight was reached under these conditions. The dried filter papers were stored in a desiccator. To measure fungal growth, filter paper was weighed and 100 ml of the culture was filtered through the filter paper on a Buchner funnel. The cells were washed twice with distilled water. The filter paper containing the growth was dried at 100°C

under vacuum 30 inch for 5 h to reach constant weight.

Evaluation of Lipase Activity

1. Silica Gel Assay Method. Lipase activity in cell free broth of Penicillium caseicolum was assayed by silica gel chromatographic method. The technique was established by Harper et al. (1956) and modified by Chandan (1962). Free fatty acids liberated by the action of lipase on substrate are extracted chromatographically and titrated by a standard alcoholic KOH.

Materials and Reagents

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

b. Silicic acid: 100 mesh powder.

c. 2 M phosphotase buffer pH 6.4. Stock solution of 2 M NaH_2PO_4 (27.2 g/100 ml) and 2 M Na_2HPO_4 (34.8 g/100 ml) were prepared and two solutions mixed to obtain pH 6.4.

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

e. Eluant: Five parts of n-butanol in 95 parts of chloroform (v/v).

f. Titrating reagent. 0.01 N ethanolic KOH.

g. Phenol red indicator (0.1%). Phenol red (100 mg) was ground in 0.1 ml of 0.1N KOH and made to 100 ml with absolute ethyl alcohol.

h. 20% (V/V) sulfuric acid.

Preparation of Assay Sample

The substrate buffer oil (20%) was mixed with 10% gum arabic solution and homogenized at 60-65°C five times in a hand homogenizer. The pH of the emulsion was adjusted to 6.5.

Five milliliters of the substrate and 5 ml of cell-free broth were mixed and incubated at 25°C for 2 h. Following incubation the enzyme-substrate mixture was acidified to pH 1.8-2.0 with 0.3 ml of 20% H_2SO_4 to stop the reaction. Silica gel (18-20 g) was added to the reaction mixture and thoroughly mixed and ground.

Preparation of Chromatographic Silicilic Acid Column

The column contained two sections:

a. Bottom Section. The column was attached to 500 ml suction flask. A filter paper disc (Whatman #5) was placed on the fritted glass disc bottom of the column. Silica gel slurry (25 ml) was placed on the top of this section. Another filter paper disc was placed over the slurry.

b. Top Section. Silica gel-acidified lipolyzed sample was slurried in 50 ml of 5% n-butanol in chloroform, then transferred quantitatively to the top of the column. This

was repeated two times with 50 ml each of the eluant. In order to extract the free fatty acids, suction was applied to give an eluate rate of 30 ml/min.

After elution, 0.3 ml of the phenol red indicator and 15 ml of absolute alcohol were added to the eluate. The eluate was titrated with 0.01 N alcoholic KOH. The titer value was corrected for the initial free fatty acid content by extraction of control sample containing cell free broth (enzyme) and substrate without incubation.

Lipase Activity

The activity of lipase was expressed as micromoles of free fatty acids liberated/5 ml of cell free broth of Penicillium caseicolum.

The results expressed in this dissertation represent average values for a mimimum of 3 trials, unless otherwise indicated.

2. pH-Stat Method

pH-Stat equipment was composed of four basic modules:

1. Titrator (E526 Metrohm Herisau)
2. Motor Drive Piston Burette (E525 Metrohm Herisau)
3. Potentiometer Recorder (Servogor 210)
4. Constant temperature circulator Model 80 (Fisher Scientific Company).

The substrate was an emulsion of 10% butter oil or tributyrin in 10% gum arabic solution. The substrates were

prepared at 60-65°C and homogenized 5 times with a hand homogenizer. The pH value was adjusted to desired pH with 0.1 N NaOH. The pH-stat was standardized at the desired pH and temperature. Five milliliters of the substrate were placed in the reaction vessel and allowed to equilibrate for 2-3 min. Cell free broth (0.2 ml) of Penicillium caseicolum was then added to the substrate. As a result of lipase action, free fatty acids were liberated in the reaction mixture. Accordingly, the pH tended to drop below the set value. However, it was automatically adjusted to the set pH with 0.02 N NaOH solution. The amount of alkali utilized with respect to reaction time was registered in the potentiometric recorder chart. The time for the test was varied from 5-10 min and the slope of the chart curve was used in calculation of lipase activity. The lipase activity was expressed as micromoles of free fatty acids or butyric acid liberated/min. Lipase production was expressed as micromoles of free fatty acids/0.2 cell free broth/min.

There are some advantages of the pH-stat over silica gel method. In the pH-stat method, no extraction of free fatty acids is necessary and no manual adjustment of pH is required. Also no indicator color change is required to be detected. It is a direct method for determination of lipase kinetics as initial velocities are measured (Brockerhoff and Jensen, 1974). According to Parry et al. (1966) this method is more sensitive than silica gel method. It has some disadvantages.

However, lipase activity at pH 7.00 is difficult to measure due to incomplete titration of fatty acids with low dissociation constants. Also there is interference with titration from a buffer or protein present in the reaction mixture. This method was used for characterization of the lipase under various conditions as well as for assaying the lipase activity of various cell free extracts. The assay pH was 9.0 to insure complete titration of butyric acid.

Characterization of Lipase

Several experiments were conducted to characterize the lipase of Penicillium caseicolum. The mold was grown in 100 ml mycological broth containing 1% corn oil under shaking condition at room temperature (25 ± 1°C, 120 rpm) for 3 days. The growth was harvested and the cell-free broth was assayed for lipase by the pH-stat method described above.

a. Optimum temperature. Temperatures tested were: 25, 30, 35, 40, 45, 50 and 55°C for determining the optimum temperature of lipase activity. The pH of substrate was 9.0 and 0.2 ml of cell-free broth was added to 5 ml of the substrate. The substrate contained 2 mM $CaCl_2$. Both butter oil and tributyrin were used as substrates.

b. Optimum pH. The investigation was conducted at optimum temperature of 35°C and pH was varied from 5.0-11.0. The substrates were butter oil and tributyrin.

c. Relationship of lipase activity and enzyme concentration. The reaction was carried out using butter oil and tributyrin as substrate at 35°C and pH 9.0. The enzyme concentration varied from 0-0.8 ml.

d. Addition of certain salts. In this experiment 1-10 μmoles of each of sodium taurocholate, sodium desoxycholate and $CaCl_2$ were added individually or in combinations to the reaction mixture. The substrates were butter oil and tributyrin. The temperature of reaction mixture was 35°C and the pH was 9.0.

e. Thermostability of the enzyme. Table 8 outlines the time-temperature used to determine stability of the lipase. The cell-free broth obtained from 3 days of growth (.2 ml) was added to the reaction mixture maintained at 35°C and pH 9.0. The pH-stat Method was used to determine the enzyme activity.

Substrate Specificity

Lipase enzyme (cell free broth) was obtained after 3 days of growth in mycological broth containing 1% corn oil. The growth was carried out under submerged condition at 120 rpm and at 25 ± 1°C. The lipase activity was determined by pH-stat method. Substrate contained 10% natural and synthetic lipids. The pH of the reaction mixture was maintained at 9.0 and at 35°C. The assay was carried also by the addition of 8 μmole sodium taurocholate and 8 mM

Table 8. Time-temperatures used in the determination of stability of Penicillium caseicolum lipase.

Temperature (^{o}C)	Time
-26.6	0, 1, 2, 3, 7 and 30 days
-15	0, 1, 2, 3, 7 and 30 days
0	0, 1, 2, 3 days
4-5	0, 1, 2, 3 days
25	0, 6, 12, 18, 24, 48 and 72 h
37	0, 6, 12, 18, 24, 48 and 72 h
45	0, 6, 12, 18, 24 and 48 h
55	0, 6, 12, 18 and 24 h
62.7	30 min
100	2, 4 and 6 min
121	15 min

$CaCl_2$ to the substrate. The lipase activity was calculated as μmole free fatty acids liberated/0.2 ml cell free broth.

Concentration of the Enzyme

To the cell free broth containing the enzyme, ammonium sulfate was added to 70% saturation at 4°C and held for 2 h to ensure complete precipitation. The suspension was centrifuged at 11,000 rpm for 2 h at 4°C in Sorvall RC 2B Automatic Refrigerated Centrifuge (Dupont Co., Newtown, CT). The pellet was dissolved in 20 ml distilled water. The concentrated material was dialyzed in a cellulose acetate dialysis tubing with a molecular weight cut off 16,000-18,000 daltons. The sealed tubing was placed in cold, distilled, demineralized water, stirred and kept at 4°C for 24 h. The water was changed after 8 h. The concentrated enzyme was kept frozen.

Protein Determination

Protein in the concentrated enzyme preparation was determined by Bio-Rad protein assay method (Bio-Rad Laboratories). This colorimetric method was developed by Bradford (1976). An acidic solution of Coomassie brilliant blue G-250 was used to bind the protein. The maximum absorbance for the dye solution shifts from 465 nm to 595 nm following binding to the protein (Resisner et al., 1975; Sedmak and Grossberg, 1977). A lyophilzed bovine gamma globulin was used to prepare the standard protein solution.

The absorbance of the protein dye mixture was read at 595 nm in a spectronic 20 spectrophotometer (Bausch and Lomb), following the color development for 10 min. In some stages of this investigation, protein in the dry cell weight was determined by Microkjeldahl procedure (AOAC, 1970).

Procedure for Making Butyl Esters for GLC Analysis

Butter oil substrate emulsion containing 10% fat was prepared as described earlier. The pH was adjusted to 9.0 with 0.1 N NaOH. The pH stat method was used for enzyme substrate reaction. The reaction mixture consisted of 9.0 ml substrate and 1.0 ml concentrated enzyme. The mixture was incubated at 35°C for 15 min and acidified to pH 1.9 with 50% H_2SO_4. The free fatty acids were isolated by a column chromatography procedure used by Blakely (1970). Butyl esters of the free fatty acid were prepared according to Supelco method (1979). The butyl esters were chromatographed on HP5840A gas chromatograph equipped with flame ionization detector (FID) and a Hewlett Packard 18850A GC Terminal was used for the analysis of the fatty acid butyl esters. The glass column (2 m x 2 mm i.d.) was packed with 15% diethylene glycol succinate (DEGS) on Chromosorb W80/100 mesh with acid wash. The instrument was operated under the following conditions:

Initial temperature (T_I)	40°C
Time at T_I (t_I)	0 min

Rate of temperature increase	8°C/min
Final temperature (T_2)	185°C
Time at T_2	25 min
Injection temperature	210°C
FID temperature	350°C
Chart speed	1 cm per min
Attenuation	10
Nitrogen carrier gas	30 ml per min
Hydrogen flow rate	30 ml per min
Air flow rate	200 ml per min

Standard fatty acid butyl esters were prepared under identical conditions and used for identification of samples. Fatty acids were identified by their retention time as compared to standards.

Procedure for Making Methyl Esters for GLC Analysis

Butter oil was transesterified according to the procedure described by Shehata et al. (1970) as follows:

One milligram of butter oil was dissolved in 2 ml petroleum ether and the solvent was evaporated under a stream of nitrogen. The transesterifying reagent contained 1.5 ml 0.5N $NaOCH_3$ in methanol, six ml of petroleum ether and 2.5 ml diethyl ether. Transesterifying reagent (25 µl) was added to the vial and quickly capped. It was shaken gently to insure complete mixing, and left at room temperature for 2 min. Then 25 µl petroleum ether was added

quickly to wash the inner wall of the vial, capped immediately and left for 2 min at room temperature. The reaction mixture was ready for direct injection into GLC.

Standard fatty acid methyl esters were prepared according to the procedure of Morrison and Smith (1964) and used for identification of fatty acids.

RESULTS AND DISCUSSION

Culture Media

In the first phase of this investigation, an experiment was conducted to determine the best media for growth of Penicillium caseicolum Cl. Five fungal media were chosen for this experiment. The growth was carried out under stationary conditions at 23 ± 1°C for 4, 7, 10 and 14 days. The growth was evaluated by dry cell weight measurements. Results are presented in Figure 2. Under the conditions studied, the best growth media for Penicillium caseicolum was Czapek Dox broth, followed by mycological broth, Sabouraud maltose broth, Sabouraud Liquid medium, and malt extract broth. The variation in growth of Penicillium caseicolum appears to be mainly due to the availability of nutrients for growth and synthesis activity. Czapek-Dox broth contains carbohydrate, inorganic nitrogen and minerals. On the other hand mycological broth contains organic nitrogen, soytone. Soytone is a product of an enzymatic hydrolysis of soybean meal. The other media contain neopeptone and a source of carbon. The composition of soybean meal and neopeptone are presented in Table 9.

It appears that soybean meal contains more nutrient to support Penicillium caseicolum growth than is provided by

Figure 2. Growth of *Penicillium caseicolum* in different fungal media under stationary conditions at 23 ± 1°C.

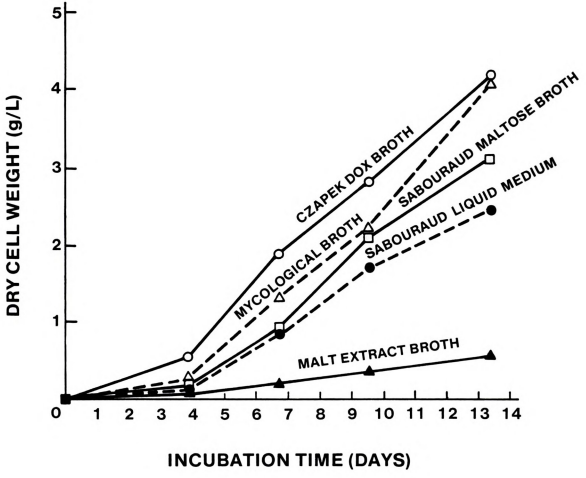

DRY CELL WEIGHT (g/L)
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
INCUBATION TIME (DAYS)
CZAPEK DOX BROTH
MYCOLOGICAL BROTH
SABOURAUD MALTOSE BROTH
SABOURAUD LIQUID MEDIUM
MALT EXTRACT BROTH

Table 9. Composition of soy meal and neopeptone.

	Soy meal[1]	Neopeptone[2] Difco
Total Nitrogen %	9.2	14.3
NaCl	4.4	-
Na	-	0.45
Ca	0.05	0.198
Fe	0.02	0.0041
Cl	-	0.84
K	3.99	0.85
Mg	0.19	0.051
P	0.38	0.19
S	0.39	-
Carbohydrates %	37	4
Amino Acids %		-
Arginine	4.6	-
Aspartic Acid	5.8	-
Cystine	0.5	.39
Glycine	2.8	-
Glutamic Acid	9.3	-
Histidine	1.6	-
Isoleucine	2.5	-
Leucine	3.2	-
Lysine	3.6	-
Methionine	0.6	-
Phenylalanine	3.6	-
Proline	3.4	-
Threonine	1.8	-
Tryptophan	0.7	0.73
Tyrosine	1.9	4.72
Valine	2.0	-

Table 9. (cont'd).

Vitamins mcg/g	0.35	-
Biotin	3.05	-
Choline	0.00115	-
Cyanocobalamin	0.81	-
Folic Acid	33	-
Niacin	7.6	-
Pantothenic Acid	4.0	-
Pyridoxine	4.3	-
Riboflavin	1.9	-
Thiamine	5.4	-

[1]Source: BBL Manual (1973) p. 163, Cockeysville, MD.

[2]Difco Manual (1972) p. 265, Detroit, MI.

-Not reported.

neopeptone. Czapek-Dox broth has most of the nutrient requirements for growth. These results are in agreement with the reports of several workers that Czapek Dox broth is an excellent medium for fungal growth (Raper and Thom, 1949). Nevertheless, mycological broth is shown by this work to be a reasonably good medium from the standpoint of the growth of the organism in stationary state. Accordingly, both Czapek-Dox broth and mycological broth were selected for lipase production studies.

Growth and Lipase Production of Various Strains of Penicillium caseicolum

Table 10 shows results of growth, lipase production and spore count of different Penicillium caseicolum strains. It was noticed that on the average pH of media dropped from 7.1 to 4.87 with different strains. Growth of different strains ranged from 8.146 to 12.092 g/l. The variation in the growth among the strains was very little except in strain K5 which showed appreciable lower growth. The spore counts were 8.4×10^6 to 1×10^7/ml. There was no relationship between the growth and spore count. For example, strain C2 had lowest spore count, but showed highest growth.

Lipase activity was determined by pH-stat method. Butter oil and tributyrin were used as substrates. The highest lipase activity was with strain B5, followed by strains C1, C2, Italian source and K5. Strain B5 had lipase activity 3-16 times more than the other strains. The results indicate no relationship between the lipase production and growth.

Little information about spore count of Penicillium caseicolum is available in the literature. In this work the spore count was determined after 1 wk of growth in Czapek-Dox agar slants, using a Haemocytometer. The spore counts through all stages of this investigation ranged from 1.16×10^7 to 1.44×10^7 spores/ml. Eitenmiller

Table 10. Growth, lipase production and spore count of various strains of Penicillium caseicolum.

Penicillium caseicolum strain	Lipase activity µmoles free fatty acids/0.2 ml/min			Growth Dry cell weight g/l	Spore count /ml
	Butter oil	Butter oil + 8 µM sodium taurocholate + 8 mM $CaCl_2$/ml	Tributyrin		
C_1	2.165	3.511	6.367	11.745	9.2×10^6
C_2	2.360	3.439	6.135	12.092	8.0×10^6
B_5	4.500	6.867	17.079	11.597	8.4×10^6
K_5	0.367	0.720	1.060	8.146	9.6×10^6
Italian source	0.950	1.229	4.083	11.073	1×10^7

et al. (1970) used $2x10^7$ spores/ml in Penicillium roqueforti work. Chander et al. (1980, 1981) inoculated Penicillium chrysogenum and Rhizopus nigricans at a rate of 2% of $1x10^7$ spores/ml.

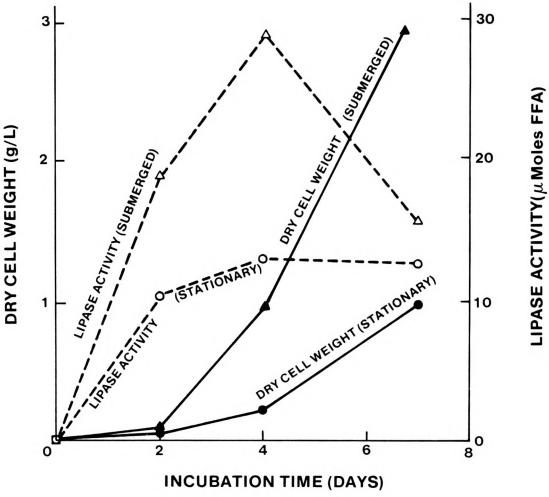
Influence of Stationary and Submerged (Shaking) Conditions on Penicillium caseicolum Growth and Lipase Production

Czapek-Dox broth and mycological broth were employed in this investigation. Growth was evaluated by dry cell weight and lipase production by silica gel chromatographic method.

In Czapek-Dox broth (Figure 3), under stationary state the growth of Penicillium caseicolum increased during incubation time, but lipase activity reached maximum after 4 days and increased very little after 7 days. In submerged (shaking) culture, relatively more growth was observed as compared to that in stationary state. The lipase activity was highest after 4 days of growth, then declined after 7 days.

The lipase activity was 13.0 and 12.7 μmoles FFA/5 ml cell free broth after 4 and 7 days of culturing under stationary condition. For the same incubation period under submerged condition, lipase activity was much higher (29.49 and 15.74 μmoles FFA/5 ml enzyme). There was decrease in lipase production after 4 days in submerged (shaking) culture, but it increased very little in stationary condition.

Figure 3. Growth and lipase production of Penicillium casei-colum in Czapek-Dox broth under stationary and submerged conditions at 25 ± 1°C and 120 rpm. Lipase activity is expressed as micromoles of free fatty acids liberated/5 ml enzyme/2 h.


DRY CELL WEIGHT (g/L)
LIPASE ACTIVITY(μ.Moles FFA)
INCUBATION TIME (DAYS)
LIPASE ACTIVITY (SUBMERGED)
LIPASE ACTIVITY (STATIONARY)
DRY CELL WEIGHT (SUBMERGED)
DRY CELL WEIGHT (STATIONARY)
0
1
2
3
0
10
20
30
2
4
6
8

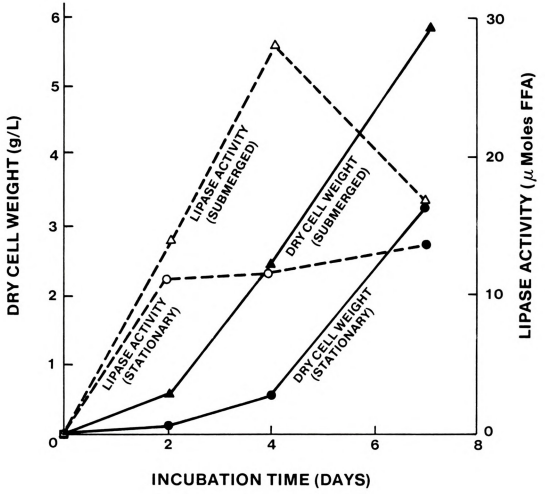
In mycological broth, growth and lipase production of Penicillium caseicolum in both phases are presented in Figure 4.

In stationary condition growth was 0.56 g/l and 3.26 g/l after 4 and 7 days, respectively. In the same period of time in submerged culture, the growth was higher (2.45 g/l and 5.84 g/l). The growth was higher 4.4 and 1.8 times after 4 and 7 days, respectively in submerged culture than in stationary culture. The lipase production was 11.62 and 13.67 μmole FFA/5 ml cell free broth after 4 and 7 days, respectively in stationary culture. For the same period in submerged culture, lipase production was 28.10 and 16.8 μmole FFA/5 ml of cell free broth. The lipase activity was appreciably higher in submerged culture than stationary culture.

The higher growth observed in submerged culture reflects the aerobic nature of the mold. The results obtained are in agreement with many workers (Calam, 1969; Belloc et al., 1975; Wang et al., 1979; Weete et al., 1974, Weete and Weber, 1980).

There was no substantial difference between lipase activity produced in mycological broth and Czapek Dox broth. The highest lipase production was obtained in both media after 4 days, declining after 7 days in submerged culture. The loss in lipase activity may be due to storage inactivation of the enzyme.

Figure 4. Growth and lipase production of Penicillium caseicolum in mycological broth under stationary and submerged conditions at 25 ± 1°C and 120 rpm. Lipase activity is expressed as micromoles of free fatty acids liberated/5 ml enzyme/2 h.


DRY CELL WEIGHT (g/L)
LIPASE ACTIVITY (μ Moles FFA)
INCUBATION TIME (DAYS)
LIPASE ACTIVITY (SUBMERGED)
DRY CELL WEIGHT (SUBMERGED)
LIPASE ACTIVITY (STATIONARY)
DRY CELL WEIGHT (STATIONARY)
0
1
2
3
4
5
6
0
2
4
6
8
0
10
20
30

Influence of Lipids Addition to the Growth Media on Lipase Production of Penicillium caseicolum

The effect of oil addition to Czapek Dox broth on lipase production of Penicillium caseicolum is presented in Figure 5. In the absence of an oil, the lipase production increased during first 4 days of growth after which it declined. Addition of oils to the medium resulted in lower lipase production. As compared to control (without oil) the relative decrease in lipase production of Penicillium caseicolum by oil addition is shown in Table 11. Considering lipase production without oil addition as 100 after 4 days, lipase production was 11.7, 45.6 and 71.7, respectively when butter oil, corn oil and olive oil were added to the medium. Apparently, the fatty acid composition of oil may be involved in this effect.

In mycological broth the observations are shown in Figure 6. Addition of butter oil, corn oil and olive oil showed a stimulatory effect on lipase production. The lipase reached maximum activity after 4 days and declined almost to the same level as with butter oil and corn oil. The enzyme was higher with olive oil at 7 days of growth.

The relative increase in lipase production by oil addition to mycological broth is listed in Table 12. Corn oil addition gave the highest increase in lipase production, followed by olive oil and butter oil. The dramatic stimulatory effect may be due to the linoleic acid content of

Figure 5. Lipase production by Penicillium caseicolum in Czapek-Dox broth containing various oils, under submerged conditions at 25 ± 1°C and 120 rpm. Lipase activity is expressed as micromoles of free fatty acids liberated/5 ml enzyme/2 h.

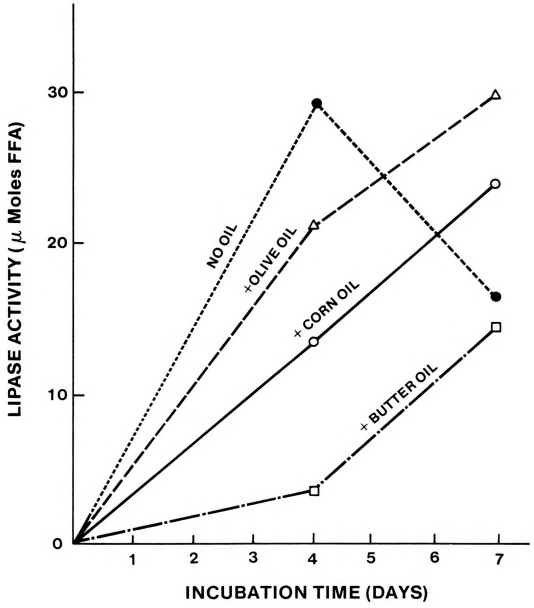

LIPASE ACTIVITY (μ Moles FFA)
30
20
10
0
1
2
3
4
5
6
7
INCUBATION TIME (DAYS)
NO OIL
+OLIVE OIL
+ CORN OIL
+ BUTTER OIL

Table 11. Influence of oil addition to Czapek-Dox broth on lipase production of Penicillium caseicolum after 4 days of growth under submerged condition.

Medium	Lipase activity µmoles FFA/5 ml/2 h	Relative lipase production
Czapek Dox broth	29.46	100
Czapek Dox broth + butter oil	3.46	11.7
Czapek Dox broth + corn oil	13.43	45.6
Czapek Dox broth + olive oil	21.13	71.7

Figure 6. Lipase production by Penicillium caseicolum in mycological broth containing various oils under submerged conditions at 25 ± 1°C and 120 rpm. Lipase activity is expressed as micromoles free fatty acids liberated/5 ml enzyme/2 h.

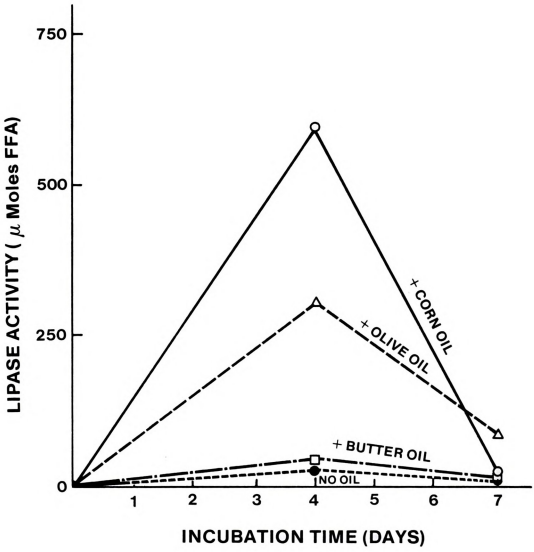

750
500
250
0
LIPASE ACTIVITY (μ Moles FFA)
+ CORN OIL
+ OLIVE OIL
+ BUTTER OIL
NO OIL
1
2
3
4
5
6
7
INCUBATION TIME (DAYS)

Table 12. Influence of oil addition to mycological broth on lipase production of *Penicillium caseicolum* after 3 days of growth.

Media	Lipase activity µMoles FFA/5 ml/2 h	Relative lipase activity
Mycological broth	36.11	1
Mycological broth + corn oil	769.26	21.3
Mycological broth + olive oil	341.18	9.4
Mycological broth + butter oil	74.96	2.1

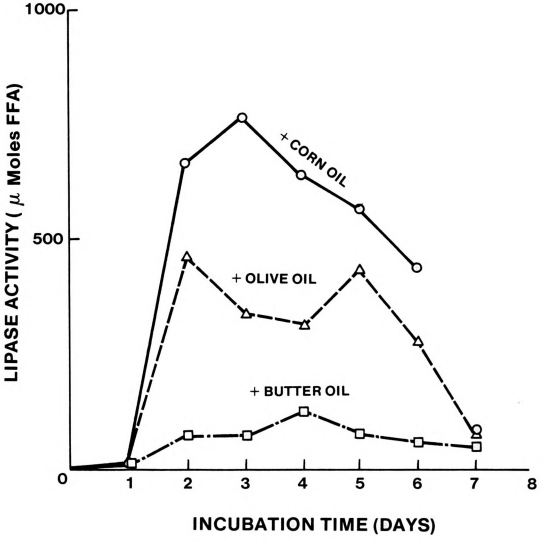
the oil. Corn oil has 53% linoleic acid. Olive oil and butter oil contain 7% and 3% linoleic acid, respectively.

Several workers found addition of lipids to the growth medium enhance lipase production of many microorganisms (Nashif and Nelson, 1953a; Khan et al., 1967; Ota et al., 1968; Yoshida et al., 1968; Iwai et al., 1973; Akhtar et al., 1974; Umemoto and Sato, 1978; Chander et al., 1979). On the other hand, some investigators observed the inhibitory effect on lipase production (Smith and Alford, 1966; Eitenmiller et al., 1970; Jonsson and Snygg, 1974; Chander et al., 1980, 1980a).

In the next phase of this study, lipase production was investigated on a daily basis using mycological broth containing either butter oil, corn oil or olive oil. The results are shown in Figure 7. Lipase production was highest in the presence of corn oil after 3 days of growth. When butter oil was used, highest lipase production was observed after 4 days of growth. Using olive oil in the medium, the lipase activity was highest on the second and fifth days of Penicillium caseicolum growth. These results indicated that stimulation of lipase production varied widely with the type of oil in the medium. Under the experimental condition, corn oil stimulated the enzyme production more than olive oil or butter oil.

It was noticed, after 3 days of growth, the pH of media dropped from 7.1 to 6.44, 5.92 and 5.83 when butter oil, olive oil and corn oil were added to the growth medium.

Figure 7. Lipase production by Penicillium caseicolum on a daily basis, in mycological broth containing various oils, under submerged condition at 25 ± 1°C and 120 rpm. Lipase activity is expressed as micromoles of free fatty acids liberated/5 ml enzyme/2 h.


1000
500
0
LIPASE ACTIVITY (μ Moles FFA)
+ CORN OIL
+ OLIVE OIL
+ BUTTER OIL
1
2
3
4
5
6
7
8
INCUBATION TIME (DAYS)

Mycological broth contains soytone as a source of nitrogen. Czapek-Dox contains inorganic nitrogen and additional minerals. A study was conducted to include soytone in Czapek-Dox broth to investigate its effect on lipase production. Results are presented in Figure 8. It may be seen that addition of soytone to Czapek Dox broth increased lipase production from 13.43 to 452.65 μmoles after 5 days of growth. However, the addition of soytone to Czapek Dox broth resulted in only 61% increase in lipase production as compared to the enzyme production in mycological broth after 4 days with corn oil. The stimulatory affect could be due to the carbohydrates, minerals and vitamins in soytone. The relative increase in lipase production as a result of soytone addition is shown in Table 13. It appears that addition of soytone and corn oil to Czapek-Dox broth increased the lipase production 13.5 times. In case of olive oil and soytone addition, the increase in the lipase production was 11.1 times. Butter oil and soytone stimulated lipase production only 2.90 times.

Corn oil was the most effective oil in lipase production of Penicillium caseicolum in mycological broth and in Czapek-Dox broth supplemented with soytone. A comparison of lipase production monitored on daily basis in both media is shown in Table 14. Lipase production in mycological broth was higher in the first 5 days. On last 2 days of incubation lipase production decreased considerably.

Figure 8. Lipase production by Penicillium caseicolum on a daily basis in Czapek-Dox broth containing various oils and soytone, under submerged condition at 25 ± 1°C and 120 rpm. Lipase activity is expressed as micromoles of free fatty acids liberated/5 ml enzyme/2 h.

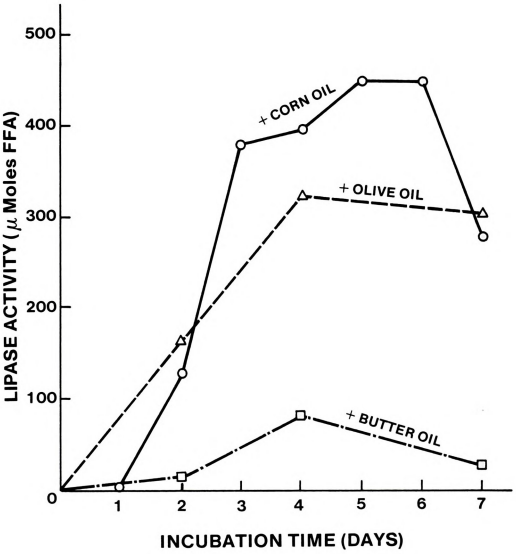

500
400
300
200
100
0
LIPASE ACTIVITY (μ Moles FFA)
+ CORN OIL
+ OLIVE OIL
+ BUTTER OIL
1
2
3
4
5
6
7
INCUBATION TIME (DAYS)

Table 13. Influence of addition of soytone to Czapek Dox broth on lipase production of *Penicillium caseicolum* (after 4 days growth).

Media	Lipase activity µmoles FFA/5 ml/ 2 h	Relative lipase production
Czapek Dox broth without oil	29.46	1
Czapek Dox broth + Soytone + corn oil	398.55	13.5
Czapek Dox broth + Soytone + olive oil	326.40	11.1
Czapek Dox broth + Soytone + butter oil	85.48	2.9

Table 14. Comparison of lipase production of *Penicillium caseicolum* on daily basis in Czapek Dox broth and mycological broth.

Incubation time, days	Czapek Dox broth + soytone + corn oil	Mycological broth + corn oil
	Lipase activity, µmoles FFA/5 ml/2 h	
1	0.72	14.81
2	129.15	669.79
3	381.97	769.26
4	398.55	634.11
5	452.26	566.42
6	452.93	436.04
7	280.38	70.8

Influence of Different Nitrogen Sources on Growth and Lipase Production of Penicillium caseicolum

The results of this study are presented in Table 15. The pH of the media dropped by 0.57 to 2.08, depending on nitrogen source. The dry cell weight (growth) was highest with soytone followed by proteose peptone, 29% whey protein concentrate; peptone, 20% whey protein concentrate, trypticase, mycological broth without oil, casamino acid, casein and sodium caseinate. The growth differences seem to reflect varying degree of amino acid uptake from different sources of nitrogen. The protein content in the growth medium appeared to be approximately related to the growth pattern.

Lipase activity was highest with soytone, followed by peptone, trypticase, proteose peptone, sodium caseinate, 29% whey protein concentrate, casein, casamino acid, 20% whey protein concentrate and mycological broth without oil.

It may be concluded that soytone supports excellent growth and best lipase production by Penicillium caseicolum. Also there was no relationship between the level of growth and lipase production.

Several workers found peptone to be a good source of nitrogen for lipase production by certain microorganisms (Imamura and Kataoka, 1963; Hosono and Tokita, 1970; Chander et al., 1977, 1980a; 1981). In this investigation, soytone and peptone were generally comparable in their effect. Soytone produced slightly higher lipase than peptone.

Table 15. Influence of different sources of nitrogen on *Penicillium caseicolum* growth and lipase production after 3 days (submerged condition) at 25 ± 1°C and 120 rpm.

Medium	Dry weight cell g/l	Protein in fungal growth mg/l	Lipase activity µMoles/ FFA/5 ml/ 2 h	Relative lipase activity %	Drop in pH
Soytone	10.393	2247.00	702.19	100	1.03
Peptone	8.805	1966.00	689.52	98.20	1.03
Trypticase	6.496	1914.9	543.74	77.43	0.57
Proteose peptone	10.082	2029.2	539.87	76.88	1.17
Sodium caseinate	2.621	761.5	362.4	51.61	1.90
29% whey protein concentrate	8.985	1420.9	220.53	31.41	1.70
Casein	2.965	773.85	187.70	26.64	1.41
Casamino acids	3.047	915.7	158.52	22.62	1.20
20% whey protein concentrate	7.484	1533.3	75.114	10.70	2.08
Mycological broth (no oil)	3.440	946.0	36.11	5.14	1.46

Influence of Different Carbon Sources on Growth and Lipase Production of Penicillium caseicolum

Table 16 shows the effect of different sources of carbon in the medium on growth and lipase production of Penicillium caseicolum. It was noticed that pH of these media dropped 1.2 to 1.94 with different sources of carbon. The dry cell weight (growth) was highest with lactose followed by galactose, fructose, dextrose, sucrose and maltose. The protein content of dry cell weight was highest with fructose followed by dextrose, galactose, sucrose, maltose and lactose.

The lipase activity was highest with dextrose followed by sucrose, maltose, galactose, lactose and fructose. Lipase activity was 51.61, 51.32, 33.37, 30.12, 7.16 and 4.35 μmoles FFA/mg of growth with dextrose, sucrose, maltose, galactose, lactose and fructose, respectively.

Although the addition of different sources of carbon to the media increased the fungal growth in some cases, it does not necessarily increase the lipase production. No relationship between the fungal growth and lipase production was evident. However, dextrose and sucrose were the best sources of carbon for both growth and lipase production stand points. These results are in agreement with the reports of some workers. Weete and Weber (1980) stated glucose was the most important source of carbon for growth of fungi. Chander et al. (1977, 1980a, 1981) found glucose to be a good source of carbon. Certain workers found

Table 16. Influence of different carbon sources on *Penicillium caseicolum* growth and lipase production (submerged condition) at 25 ± 1°C and 120 RPM after 3 days.

Carbon source	Dry cell weight g/l	Protein in the fungal growth mg/l	Lipase activity µMole FFA /5 ml/2 h	Lipase activity µMoles FFA /mg of growth	Relative lipase activity %
Dextrose	10.103	2527.86	521.44	51.61	100
Sucrose	10.080	1770.98	517.30	51.32	99.4
Maltose	9.654	1640.87	322.20	33 37	64.7
Galactose	12.213	2416.68	367.82	30.12	58.4
Lactose	12.663	1060.25	90.72	7.16	13.9
Fructose	11.973	2899.41	52.14	4.35	8.4

glucose to inhibit lipase production (Imamura and Kataoka, 1963; Mates and Sudakevitz, 1973). Others reported no significant change in lipase production with different carbohydrates (Iwai and Tsujisaka, 1974).

Influence of Addition of Different Minerals on Penicillium caseicolum Growth and Lipase Production

Results of this study are presented in Table 17. In general, the addition of minerals to mycological broth had inhibitory effect on the lipase production. The growth of Penicillium caseicolum was not appreciably affected by the addition of minerals.

Table 17. Effect of selected minerals on Penicillium caseicolum growth and lipase production after 3 days of growth under submerged condition at 25 ± 1°C and 120 rpm.

Medium	% mineral in media	Dry cell weight g/l	Protein in fungal growth mg/l	Lipase activity µMoles FFA /5 ml cell free broth	Relative activity %
MB	-	10.977	2245.9	697.02	100.0
MB+(K_2HPO_4)	0.1	10.459	2702.8	686.75	98.5
MB+KCl	.05	10.765	2368.47	513.63	73.7
MB+$FeSO_4$	0.001	10.834	2158.4	506.21	72.6
MB+$NaNO_3$+K_2HPO_4 +$MgSO_4$+KCl+$FeSO_4$	0.3, 0.1, 0.05, 0.05, 0.001	11.066	2401.14	476.02	68.3
MB+$NaNO_3$	0.3	10.985	3379.0	457.00	65.6
MB+$MgSO_4$	0.05	11.368	2001.8	372.86	53.5
MB+$CaSO_4$	0.1	10.052	1828.18	19.27	2.8

MB=Mycological broth

Influence of pH on Penicillium caseicolum Growth and Lipase Production

The effect of pH on Penicillium caseicolum growth and lipase production was studied in a fermentor under pH-stat conditions. The lipase production was determined by pH-stat method using tributyrin and butter oil substrates with and without sodium taurocholate and $CaCl_2$ addition.

The growth of Penicillium caseicolum at different media pH levels is presented in Figure 9. In general, the lower the pH the better was the growth. On the fifth day at pH 5.5 and 6.5, the growth was higher than at pH 4.7. Lipase activity of Penicillium caseicolum against different substrates is presented in Figure 10, 11, and 12. At lower pH levels, the lipase production was very low. The lipase production increased with increase in the pH of media. However, lipase production was also low at a higher pH, such as 8.5. The lipase production reached a maximum after 4 days of growth at all pH levels except at pH 7.5 lipase production peaked at 3 days of growth. The lipase activity at pH 7.5 after 3 days of culturing was lower than at pH 7.0 after 4 days of growth. The relationship between lipase activity and pH was similar where tributyrin or butter oil was used as substrate.

These results indicated pH 7.0 is the optimum pH for lipase production by Penicillium caseicolum. The results

Figure 9. Effect of different pH on growth of *Penicillium caseicolum* at 25 ± 1°C and 120 rpm.

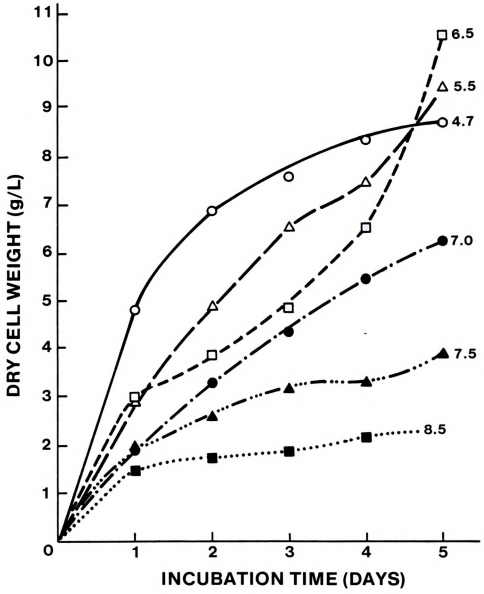

DRY CELL WEIGHT (g/L)
INCUBATION TIME (DAYS)
6.5
5.5
4.7
7.0
7.5
8.5

Figure 10. Effect of pH on lipase production by Penicillium caseicolum at 25 ± 1°C and 120 rpm. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/min, was determined by pH-stat method at pH 9.0 and temperature 35°C toward tributyrin.

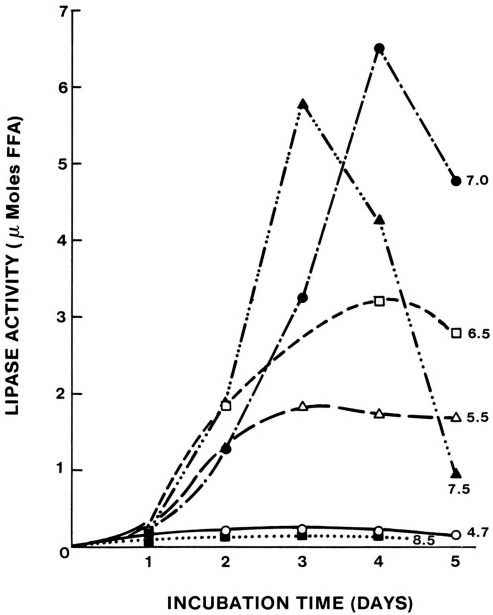

LIPASE ACTIVITY (μ Moles FFA)
INCUBATION TIME (DAYS)
0
1
2
3
4
5
6
7
7.0
6.5
5.5
7.5
4.7
8.5

Figure 11. Effect of pH on lipase production by Penicillium caseicolum at 25 ± 1°C and 120 rpm. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/min., was determined by pH-stat method at pH 9.0, and temperature 35°C toward butter oil.

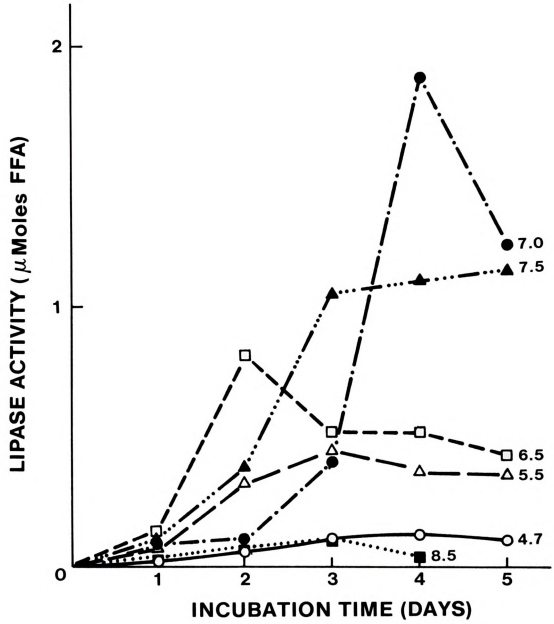
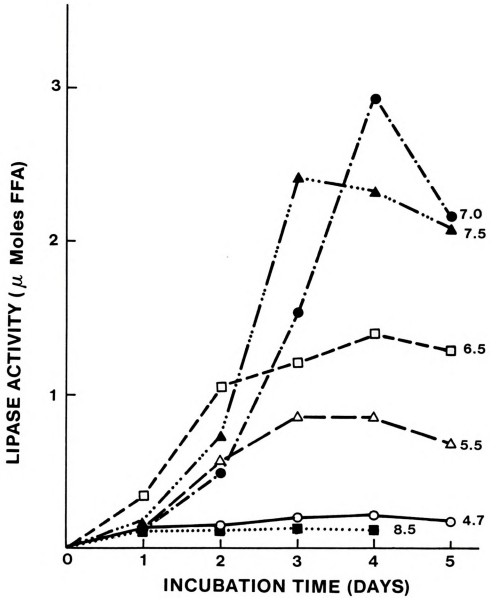

LIPASE ACTIVITY (μMoles FFA)
2
1
0
1
2
3
4
5
INCUBATION TIME (DAYS)
7.0
7.5
6.5
5.5
4.7
8.5

Figure 12. Effect of pH on lipase production by Penicillium caseicolum at 25 ± 1°C and 120 rpm. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/min., was determined by pH-stat method at pH 9.0 and temperature 35°C toward butter oil containing 8 μmole sodium taurocholate and 8 mmole $CaCl_2$/ml.


LIPASE ACTIVITY (μ Moles FFA)
3
2
1
0
1
2
3
4
5
INCUBATION TIME (DAYS)
7.0
7.5
6.5
5.5
4.7
8.5

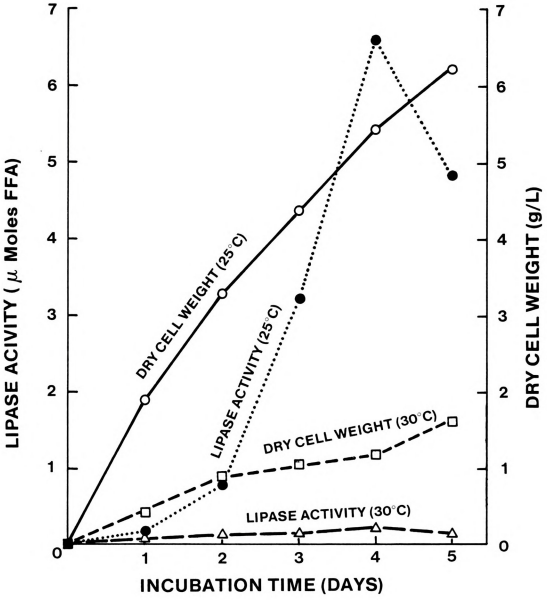
are in agreement with the data of Stephaniak et al. (1980) in that a higher pH favored lipase production by Penicillium candidum (P. caseicolum). The growth was maximum at pH 4.7, but optimum lipase production was at pH 7.0. Raper and Thom (1949) reported most Penicillium sp. grew best in a mildly acidic medium. Weete and Weber (1980) stated optimum pH for growth of most fungi is between 6.0 and 7.0. Sartory et al. (1927) reported the optimum pH of Penicillium caseicolum is at pH 6.5-7.0.

Influence of Temperature on Penicillium caseicolum Growth and Lipase Production

The effect of temperature on Penicillium caseicolum growth and lipase production is presented in Figure 13. The results indicated the maximum lipase production occurred in 4 days of culturing at 25°C. The fungal growth at 25°C was dramatically higher than at 30°C.

The lipase activity showed a similar pattern and trend with tributyrin as with butter oil with or without sodium taurocholate and $CaCl_2$. The fungal growth continued to increase at 25°C and 30°C, but the lipase activity decreased after 4 days.

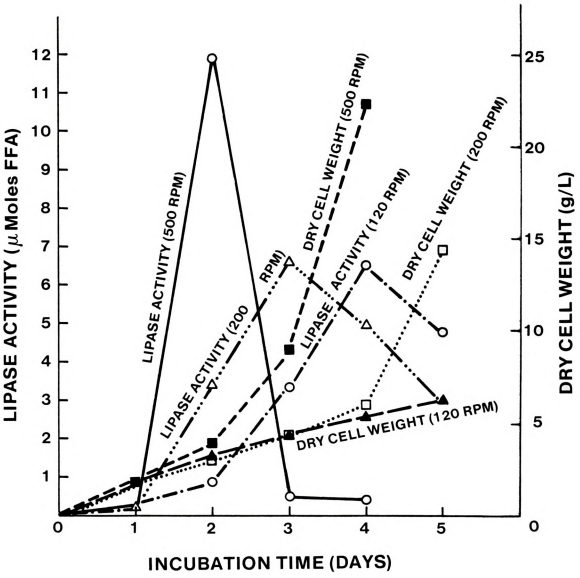
Figure 13. Effect of temperature on growth and lipase production by Penicillium caseicolum at pH 7.0, and 120 rpm. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/min., was determined by pH-stat method at pH 9.0, and temperature 35°C toward tributyrin.


LIPASE ACIVITY (μ Moles FFA)
DRY CELL WEIGHT (g/L)
INCUBATION TIME (DAYS)
DRY CELL WEIGHT (25°C)
LIPASE ACTIVITY (25°C)
DRY CELL WEIGHT (30°C)
LIPASE ACTIVITY (30°C)
0
1
2
3
4
5
6
7

Influence of Agitation on Penicillium caseicolum Growth and Lipase Production

The results are presented in Figure 14. In general, the fungal growth was higher when the agitation was increased. Highest growth was obtained at 500 rpm. Presumably, an increase in agitation enhanced the oxygen uptake and led to increased metabolic activity and growth of the fungus. The lipase activity was also highest at the agitation of 500 rpm and showed maximum activity after 2 days of growth when tributyrin was used as the substrate. A similar pattern was obtained when butter oil was used as substrate with or without the addition of sodium taurocholate and $CaCl_2$.

Figure 14. Effect of agitation on growth and lipase production by Penicillium caseicolum. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/min., was determined by pH-stat method at pH 9.0 and temperature 35°C toward tributyrin.


LIPASE ACTIVITY (μMoles FFA)
DRY CELL WEIGHT (g/L)
INCUBATION TIME (DAYS)
LIPASE ACTIVITY (500 RPM)
LIPASE ACTIVITY (200 RPM)
LIPASE ACTIVITY (120 RPM)
DRY CELL WEIGHT (500 RPM)
DRY CELL WEIGHT (200 RPM)
DRY CELL WEIGHT (120 RPM)
0
1
2
3
4
5
6
7
8
9
10
11
12
15
20
25

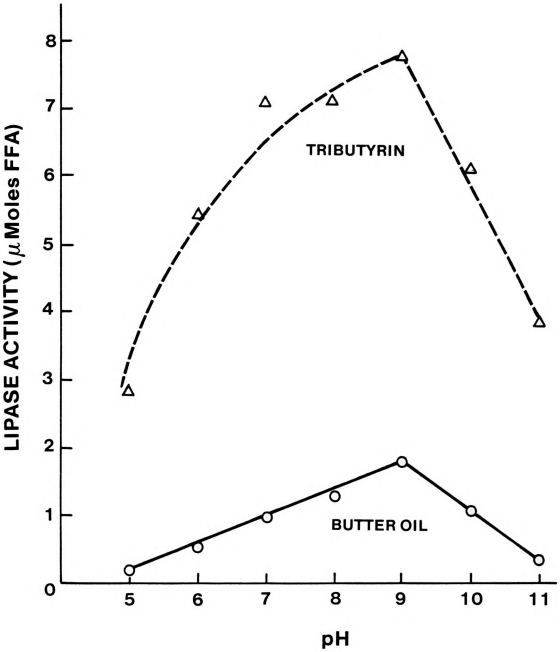
Characterization of Penicillium caseicolum Lipase

Optimum pH of Penicillium caseicolum Lipase

Optimum pH of Penicillium caseicolum lipase towards tributyrin and butter oil was determined by the pH-stat method. Results are presented in Figure 15. The optimum pH of Penicillium caseicolum lipase was at pH 9.0 toward both tributyrin and butter oil. As the pH increased or decreased in relation to the optimum pH, the rate of hydrolysis decreased. With tributyrin there was a sharper decrease in the lipase activity than with butter oil. When using butter oil as a substrate there was very little activity at pH 5 and 11.

Lambert and Lenoir (1976) reported the optimum pH of Penicillium caseicolum lipase to be around 9.0 to 9.6.

Figure 15. Effect of pH on lipase activity of Penicillium caseicolum, was determined by pH-stat method at 35°C toward tributyrin and butter oil.


LIPASE ACTIVITY (μMoles FFA)
8
7
6
5
4
3
2
1
0
TRIBUTYRIN
BUTTER OIL
5
6
7
8
9
10
11
pH

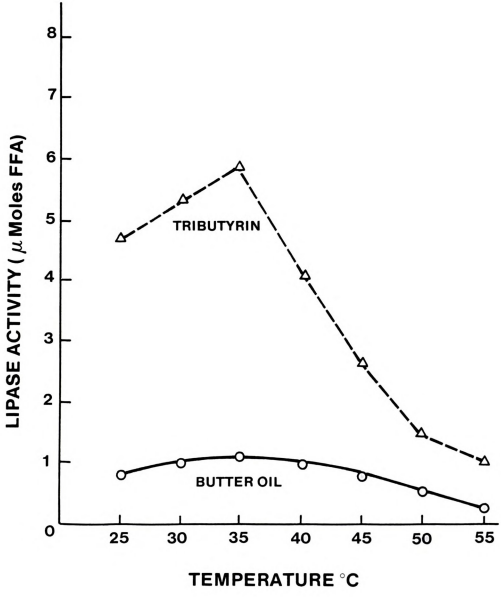
Optimum Temperature of Penicillium caseicolum Lipase

pH stat method was employed to determine the optimum temperature of Penicillium caseicolum lipase. Tributyrin and butter oil were used as substrates. The results are presented in Figure 16. The optimum temperature of the lipase activity was 35°C for both substrates. When compared to butter oil, there was very sharp decrease in the rate of hydrolysis of tributyrin as the temperature increased.

Microbial lipases have been reported to be active over a wide range of temperatures, but the optimum temperatures range is 30-40°C (Hugo and Beveridge, 1962; Tomizuka, 1966; Vadehra and Harmon, 1968; Khan et al., 1967; Umemoto et al., 1968; Eitenmiller et al., 1970; Mosona and Tokito, 1970; Collins et al., 1971; Tsujisaka et al., 1972; Iwai and Tsujisaka, 1974; Chander et al., 1979). Other investigators reported optimum temperatures for lipase of certain microorganisms to be higher than 40°C (Tomizuka, 1969; Lawrence, 1967; Somkuti et al., 1969; Oterholm et al., 1970; Liu et al., 1973). Chopra et al. (1980) has reported optimum temperature for lipase activity of Aspergillus wentii as 25°C.

Belloc et al. (1975) studied lipase activity of Penicillium camemberti at 25°C. The results obtained in this investigation are in agreement with those obtained by Lamberet and Lenoir (1975) who reported 35°C as an optimum temperature for lipase activity of purified Penicillium caseicolum lipase.

Figure 16. Effect of temperature on lipase activity of Penicillium caseicolum was determined by pH-stat method at pH 9.0 toward tributyrin and butter oil.


LIPASE ACTIVITY (μ Moles FFA)
8
7
6
5
4
3
2
1
0
TRIBUTYRIN
BUTTER OIL
25
30
35
40
45
50
55
TEMPERATURE °C

Influence of Bile Salts and Calcium Chloride on Lipase Activity of Penicillium caseicolum

The influence of bile salts and $CaCl_2$ on the lipase activity was investigated by the pH-stat method using tributyrin and butter oil as substrates. Different concentrations of sodium taurocholate, sodium desoxycholate and $CaCl_2$ individually or in mixtures were used. Cell free broth was used as the source of enzyme. When tributyrin was used as substrate (Figure 17), sodium taurocholate, sodium desoxycholate and $CaCl_2$ had inhibitory effect on lipase activity. On the other hand, when butter oil was used as substrate (Figure 18), sodium taurocholate and sodium desoxycholate had stimulatory effect on lipase activity at all concentrations. Sodium taurocholate stimulated the lipase activity more than sodium desoxycholate. However, at concentration of 10 µMole sodium desoxycholate stimulated the initial rate of hydrolysis more than sodium taurocholate did. Calcium chloride had inhibitory effect on the rate of hydrolysis, except at 2 mmole concentration where it slightly increased the rate of hydrolysis.

Mixtures of sodium taurocholate, sodium desoxycholate and $CaCl_2$ in different concentrations were studied for their effect on the rate of hydrolysis of tributyrin and butter oil. The results obtained in case of tributyrin are presented in Figure 19. Sodium taurocholate, sodium desoxycholate and $CaCl_2$ in any concentration of mixtures had

Figure 17. Effect of different concentration of sodium taurocholate, sodium desoxycholate and $CaCl_2$ on lipase activity of Penicillium caseicolum. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/ min., was determined by pH-stat method at pH 9.0 and temperature 35°C toward tributyrin.

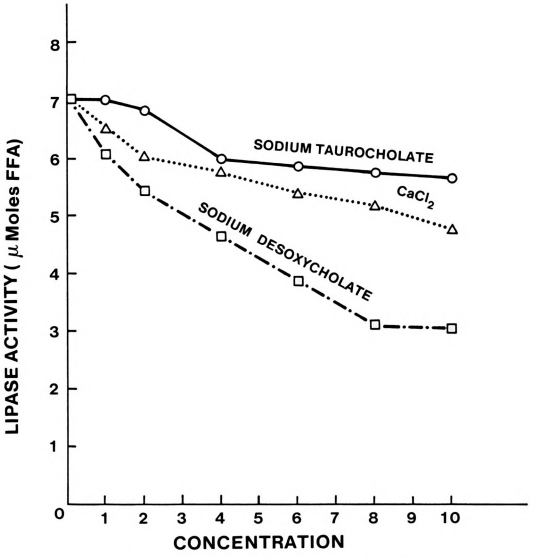

LIPASE ACTIVITY (μ Moles FFA)
0
1
2
3
4
5
6
7
8
SODIUM TAUROCHOLATE
CaCl2
SODIUM DESOXYCHOLATE
1
2
3
4
5
6
7
8
9
10
CONCENTRATION
μ Mole SODIUM TAUROCHOLATE AND DESOXYCHOLATE/ml
or mMole CaCl2/ ml

Figure 18. Effect of different concentration of sodium taurocholate, sodium desoxycholate and $CaCl_2$ on lipase activity of Penicillium caseicolum. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/ min., was determined by pH-stat method at pH 9.0 and temperature 35°C toward butter oil.

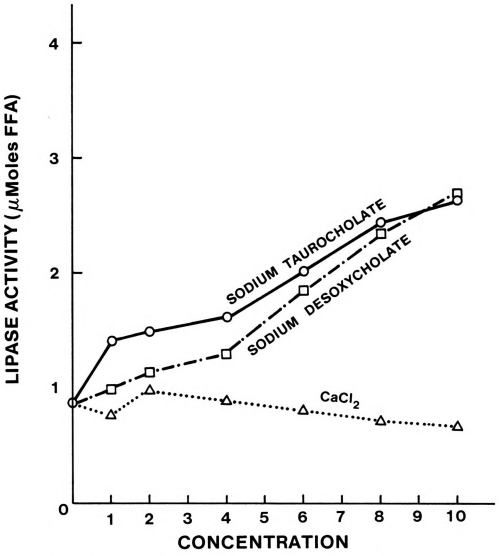
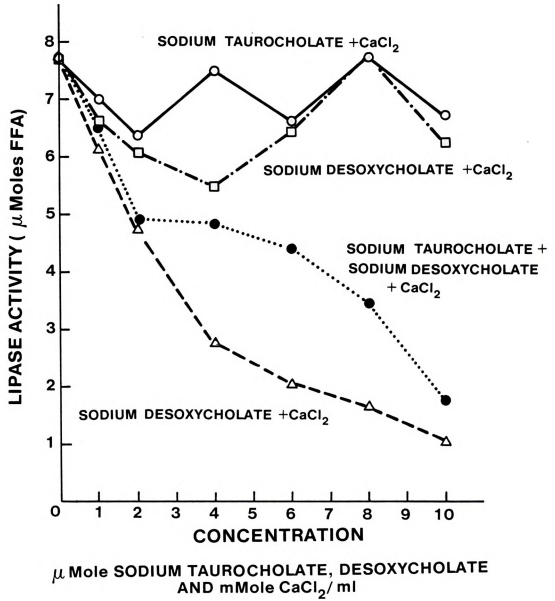

LIPASE ACTIVITY (μMoles FFA)
4
3
2
1
0
SODIUM TAUROCHOLATE
SODIUM DESOXYCHOLATE
CaCl2
1 2 3 4 5 6 7 8 9 10
CONCENTRATION
μMole SODIUM TAUROCHOLATE AND DESOXYCHOLATE/ml
OR mMOLE CaCl2/Ml

Figure 19. Effect of different mix concentration of sodium taurocholate, sodium desoxycholate and $CaCl_2$ on lipase activity of Penicillium caseicolum. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml enzyme/min., was determined by pH-stat method, at pH 9.0 and temperature 35°C toward tributyrin.

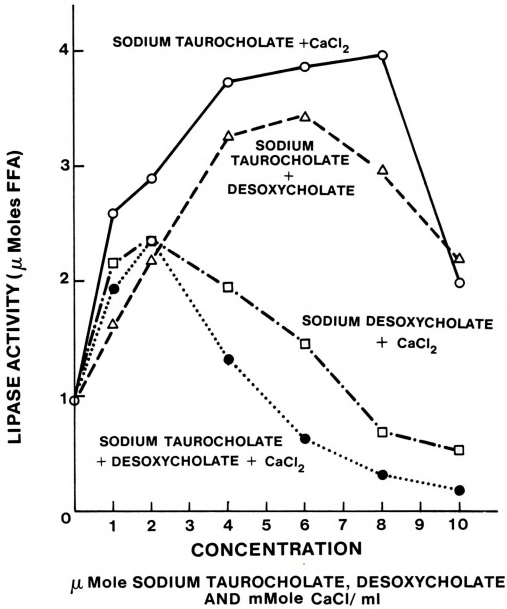

SODIUM TAUROCHOLATE +CaCl2
SODIUM DESOXYCHOLATE +CaCl2
SODIUM TAUROCHOLATE + SODIUM DESOXYCHOLATE + CaCl2
SODIUM DESOXYCHOLATE +CaCl2
LIPASE ACTIVITY (μ Moles FFA)
0 1 2 3 4 5 6 7 8 9 10
CONCENTRATION
μ Mole SODIUM TAUROCHOLATE, DESOXYCHOLATE AND mMole CaCl2/ ml

inhibitory effect on the initial rate of hydrolysis.

Figure 20 shows the effect of mixtures of these salts on the initial rate of hydrolysis of butter oil. Sodium taurocholate + $CaCl_2$ and sodium taurocholate + sodium desoxycholate + $CaCl_2$ in all concentration tested had a stimulatory effect as compared to no salts. With sodium taurocholate + $CaCl_2$, the highest increase in the initial rate of hydrolysis was at 8 µmole and 8 mmole, respectively. On the other hand sodium desoxycholate + $CaCl_2$ and sodium taurocholate + sodium desoxycholate + $CaCl_2$ had stimulatory effect at lower concentrations and inhibitory effect at higher concentration.

The results are in agreement with Belloc et al. (1975) who used sodium taurocholate in assaying Penicillium camemberti lipase. Lamberet and Lenoir (1976) used 2 mM $CaCl_2$ in the reaction mixture of Penicillium caseicolum. Lamberet and Lenoir (1976a) found Ca^{++} ions to be required for maximum rate of lipase activity of Penicillium caseicolum. The results presented in this work indicate varying effects of the addition of the salts according to the substrate used.

Figure 20. Effect of different mix concentration of sodium taurocholate, sodium desoxycholate and $CaCl_2$ on lipase activity of Penicillium caseicolum. Lipase activity expressed as micromoles of free fatty acids liberated/0.2 ml/enzyme/min., was determined by pH-stat method at pH 9.0 and temperature 35°C toward butter oil.


SODIUM TAUROCHOLATE +CaCl2
SODIUM TAUROCHOLATE + DESOXYCHOLATE
SODIUM DESOXYCHOLATE + CaCl2
SODIUM TAUROCHOLATE + DESOXYCHOLATE + CaCl2
LIPASE ACTIVITY (μ Moles FFA)
0
1
2
3
4
1
2
3
4
5
6
7
8
9
10
CONCENTRATION
μ Mole SODIUM TAUROCHOLATE, DESOXYCHOLATE AND mMole CaCl/ ml

Stability of Penicillium caseicolum Lipase

Stability of Penicillium caseicolum lipase was studied under different conditions of temperatures and storage time using pH-stat method for lipase assay. The results presented in Table 18 indicated that the lipase was stable at -26.6° and -15°C for one month. The enzyme showed no loss on storage for 72 h at 0° and 4°C. The enzyme lost considerable activity at higher temperatures. After 72 h of storage at 25°, 37° and 45°C, the residual enzyme activity was 98.7, 42.0 and 13%, respectively. At 55°C, it lost all its activity after 24 h of storage. When the enzyme was exposed to pasteurization treatment, it retained 1.0 of its activity. The enzyme lost its activity completely on boiling for 6 min. Autoclave treatment retained 1.5% of the enzyme activity.

The relative percent of remaining lipase activity at 37, 45, 55, 63 and 100°C was plotted on semi log paper, as shown in Figure 21, and the D-values were derived from the graph. The D-values are presented in Table 19. It was observed that the D-values for experimental temperatures below 37°C approached infinity.

The Decimal-Reduction-Time (DRT) curve for lipase heated at 37-100°C is presented in Figure 22. A Z_D value (the change in temperature yielding a ten fold change in D value) of 15.8°C was derived from the DRT curve.

Table 18. Stability of Penicillium caseicolum lipase as function of storage temperature and time.

Temperature °C	Storage time	Percent of residual activity
-26.6	1 month	100
-15.0	1 month	100
0	72 h	100
4	72 h	100
25	24 h	99.0
	72 h	98.7
37	18 h	98.0
	24 h	93.2
	48 h	68.0
	72 h	42.0
45	6 h	90.6
	12 h	74.0
	18 h	52.0
	24 h	38.0
	48 h	21.0
	72 h	13.0
55	6 h	1.4
	12 h	0.9
	18 h	0.5
63	30 min	1.0
100	2 min	1.6
	4 min	0.4
121	15 min	1.4

Figure 21. Time-survivor curve for Penicillium caseicolum lipase incubated at different temperature. Lipase activity determined by pH-stat method at pH 9.0, temperature 35°C toward tributyrin.

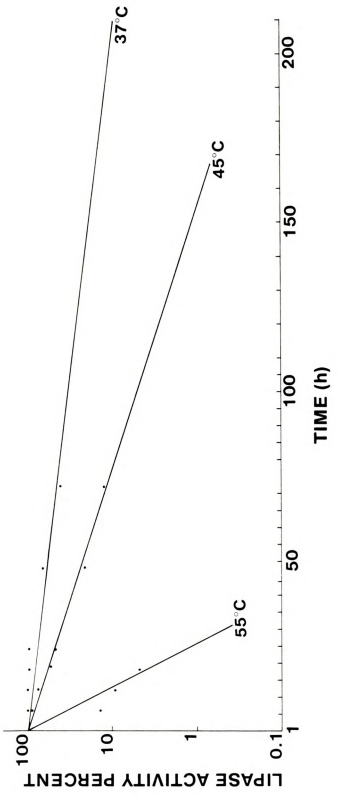
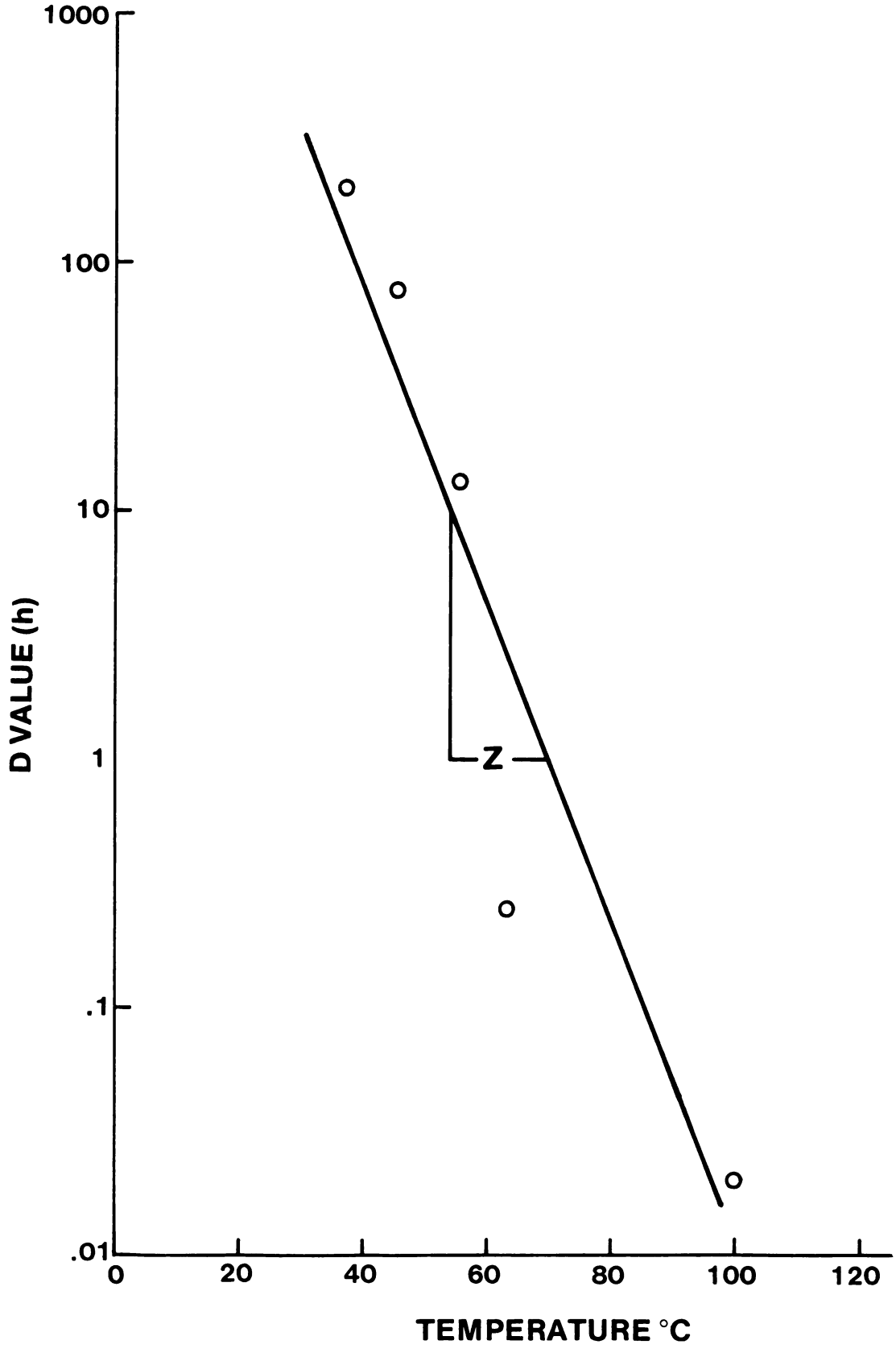

LIPASE ACTIVITY PERCENT
100
10
1
0.1
1
50
100
150
200
TIME (h)
37°C
45°C
55°C

Table 19. D-values of heat treatment of *Penicillium caseicolum* lipase at various temperature.

Temperature ^{o}C	D-value
37	200
45	78
55	13
63	0.25
100	0.02

Figure 22. Decimal-Reduction Time curve for Penicillium caseicolum lipase incubated at different temperature.


1000
100
10
1
.1
.01
D VALUE (h)
Z
0
20
40
60
80
100
120
TEMPERATURE °C

Several workers reported Z_D values for some microbial lipases. Driessen and Stadhouders (1974) found that the Z_D value of Pseudomonas fluorescens 22F lipase was 8.9°C. Kishonti (1975) reported that the Z_D value of Pseudomonas 21B lipase was 55°C. Adams and Brawley (1981) determined the Z_D value of Pseudomonas spp MC_{50} lipase to be 36°C when heated in H_2O. The lipase exhibited greatest survival at pH 8.5. Below pH 6.5 survival was less than 10% of the survival at pH 8.5.

The Z_D value of lipase could be dependent on the microorganism, strain, type of oil, oil concentration, heat treatment of substrate, composition of the substrate, method of lipase assay, temperature and pH of assay. Previous workers reported microbial lipases to be stable at lower temperature but lost significant activity at higher temperature (Nashif and Nelson, 1953; Frinkelstein et al., 1970; Chander, 1979). Various microbial lipase vary in their resistance to heat treatment. According to Anderson et al. (1979) the factors involved in the heat resistance of enzyme are primarily due to molecular structure and specific components in the molecule such as polysaccharides. Divalent cations appear to stabilize the molecule. Anderson et al. (1979) and Liu et al. (1973, 1977) stated a high content of hydrophobic amino acids in the enzyme molecule, disulfide bridges and other bonds play a role in stabilizing the enzyme molecule. Frieden (1971) reported that the inactivation of

lipase at a low temperature may be due to dissociation of monomer into subunits. The dissociated and inactived enzyme may be slowly converted to different forms. Reassociation may occur after rewarming, but the enzyme is still inactive. Cooper (1977) stated that the freezing and thawing of some proteins may decrease the stability of the enzyme. Lamberet and Lenoir (1976) reported crude Penicillium caseicolum lipase to be stable within pH 7.0 to 8.5 at 30°C. Seventy percent of initial activity was lost after 30 min of storage at 30°C at pH 6.0. Lamberet and Lenoir (1976a) studied the stability of purified Penicillium caseicolum lipase which appeared to be more stable than the crude enzyme. The purified enzyme was stable for 15 min below 35°C. The enzyme lost 60% of activity on storage for 5 min at 40°C and pH 8.5, but lost 90% of its activity when stored at 40°C for 60 min.

The results obtained in this investigation are in disagreement with the work of Lamberet and Lenoir (1976). This may be due to different strains, media composition, pH of media, and the environmental growth factors.

Substrate Specificity of Penicillium caseicolum Lipase

The effect of Penicillium caseicolum lipase activity on natural and synthetic lipids was investigated by pH-stat method. The results are presented in Table 20. The highest initial rate of hydrolysis was toward tributyrin. The relative ratesof hydrolysis were 47.3, 39.8, 33,7, 25.6, 22.9, 15.9, 9.0% with tricaproin, tricaprylin, tristearin, triolein, trilaurin, trimyristin and tripalmatin, respectively. On the other hand, the relative rate of hydrolysis in natural lipids ranged from 17.8 to 34.5% with various lipids. Previous studies show addition of 8 μmole of sodium taurocholate and 8 mmoles $CaCl_2$ to reaction mixture enhanced the rate of hydrolysis by Penicillium caseicolum lipase. A study was conducted to investigate the effect of these salts on the various lipids. The results are presented in Table 21. It appears that there were inhibitory effect with some lipids and stimulatory effects on the other lipid substrates.

In general, the results obtained show Penicillium caseicolum lipase preferentially hydrolyzed short chain fatty acid glycerides. These results are similar to the results obtained by some workers for certain microbial lipases (Umemoto et al., 1968; Otherholm et al., 1968, 1970; Angeles and Marth, 1971: Yamaguchi et al., 1973; Chander et al., 1973; Formisano et al., 1974; Chander et al., 1979a. However, Khan et al. (1967)

Table 20. Relative activity of Penicillium caseicolum lipase toward natural and synthetic lipids.

Natural and synthetic lipids	Lipase activity µmoles FFA/ 0.2 ml/min.	Relative rate of hydrolysis %
Tributyrin	6.24	100
Tricaproin	2.95	47.3
Tricaprylin	2.49	39.8
Tristearin	2.10	33.7
Triolein	1.60	25.6
Trilaurin	1.43	22.9
Trimyristin	0.99	15.9
Tripalmitin	0.56	9.0
Corn oil	2.15	34.5
Sunflower oil	2.07	33.2
Grape seed oil	1.91	30.5
Almond oil	1.82	29.2
Safflower oil	1.79	28.7
Soy oil	1.73	27.7
Peanut oil	1.73	27.7
Butter oil	1.52	24.4
Sesame oil	1.48	23.7
Lard	1.42	22.8
Hazelnut oil	1.35	21.6
Walnut oil	1.31	21.0
Olive oil	1.11	17.8

Table 21. Effect of adding sodium taurocholate and $CaCl_2$ on activity of Penicillium caseicolum lipase toward different natural and synthetic lipids.

Natural and synthetic lipid	Lipase Activity FFA/0.2 ml /min.	Lipid Activity with 8 μm sodium taurocholate and 8 mm $CaCl_2$ μmoles FFA/0.2 ml/min.	% increase (+) or decrease (-)
Tributyrin	6.24	-	-
Tripalmitin	0.56	1.18	+110.7
Tricaprytin	2.49	3.63	+ 45.8
Trilaurin	1.43	1.93	+ 35.0
Trimyristin	.99	1.33	+ 34.3
Tricaproin	2.95	3.87	+ 31.2
Tristearin	2.10	2.60	+ 23.8
Triolein	1.60	1.38	- 13.8
Butter oil	1.52	3.61	+137.5
Olive oil	1.11	2.55	+129.7
Lard	1.42	3.02	+112.7
Sesame oil	1.48	1.82	+ 23.0
Grape seed oil	1.91	1.62	- 15.2
Walnut oil	1.31	1.04	- 20.6
Hazelnut oil	1.35	0.91	- 32.6
Peanut oil	1.74	0.99	- 42.8
Soy oil	1.73	0.95	- 45.1
Safflower oil	1.75	0.96	- 54.9
Corn oil	2.15	0.88	- 59.1
Almond oil	1.82	0.67	- 63.2
Sunflower oil	2.07	0.65	- 68.6

reported that an extracellular lipase of Achromobacter lipolytica hydrolyzed triolein more rapidly than tributyrin. Oi et al. (1969) found that an intracellular lipase of Rhizopus hydrolyzed triolein more efficiently than tributyrin. The results obtained in this investigation are in agreement with those obtained by Alford et al. (1964). Oi et al. (1969), Eitenmiller et al. (1970). Belloc et al. (1975) found lipase activity of Penicillium camemberti was relatively higher toward tributyrin.

According to Lambert and Lenoir (1976), the relative activity of Penicillium caseicolum lipase toward tributyrin, butter oil and triolein was 100, 87 and 22%, respectively. In this investigation, relative lipase activity of Penicillium caseicolum toward tributyrin, triolein and butter oil was 100, 25.66 and 24.44%, respectively. These differences may be related to the strain, growth factors, method of assay, percentage of oil in the substrate, heat treatment of substrate and other components of substrate emulsion.

Concentration of Penicillium Caseicolum Lipase

Protein content and lipase activity of different fractions of Penicillium caseicolum lipase are presented in Table 22. The degree of lipase concentration was 49-fold. This concentrated enzyme was used for the specificity studies of the lipase.

Table 22. Protein content and lipase activity of different fractions of *Penicillium caseicolum* lipase.

Fraction	Total protein µg/0.2 ml enzyme	Lipase activity µM FFA/ 0.2 ml/ min	Specific activity µM FFA µg protein	Concentration
Supernatant fraction	5.91	0.07	0.012	1
Concentrated lipase	24.49	14.56	0.59	49

Fatty Acid Specificity of Penicillium caseicolum Lipase

Gas liquid chromatographic analyses of fatty acids are presented in Table 23. The table shows the free fatty acids hydrolyzed from butter oil substrate as a result of lipase action. Also the free fatty acid composition of butter oil substrate prior to lipase action and fatty acid composition of transesterified butter oil are shown. The results indicate butyric acid was preferentially released by the action of the lipase. Little or no increase in the other short chain fatty acids (C_6 and C_8) was observed. It is concluded that Penicillium caseicolum lipase has a high specificity toward butyric acid glycerides of butter oil. Typical chromatogram of free fatty acids in lipolyzed sample, free fatty acids in butter oil substrate and fatty acid composition of butter oil are presented in Figures 23. 24 and 25, respectively. The free fatty acid profiles of Penicillium caseicolum lipase shown here are generally in agreement with the work of Kornackl et al. (1979). The results also confirm the observations in the previous study of this investigation that the enzyme has a specificity for short chain fatty acids.

Table 23. Free fatty acids liberated by action of *Penicillium caseicolum* lipase and fatty acid composition of butter oil.

Fatty acid	FFA in lipolyzed butter oil substrate %	FFA in butter oil substrate %	Fatty acids composition of butter oil by transesterification %
C_4	19.01	0	3.34
C_6	1.80	1.15	2.41
C_8	1.60	0.36	1.44
C_{10}	2.25	3.72	3.79
C_{12}	4.02	4.10	4.10
C_{14}	12.74	13.62	12.10
$C_{14:1}$	6.59	7.45	2.92
C_{16}	22.05	18.82	29.25
$C_{16:1}$	7.29	12.84	4.49
C_{18}	7.98	14.06	10.67
$C_{18:1}$	12.96	17.50	22.15
$C_{18:2}$	1.54	6.39	3.34

Figure 23. Gas chromatogram of free fatty acids in lipolyzed butter oil as a result of Penicillium caseicolum lipase action.

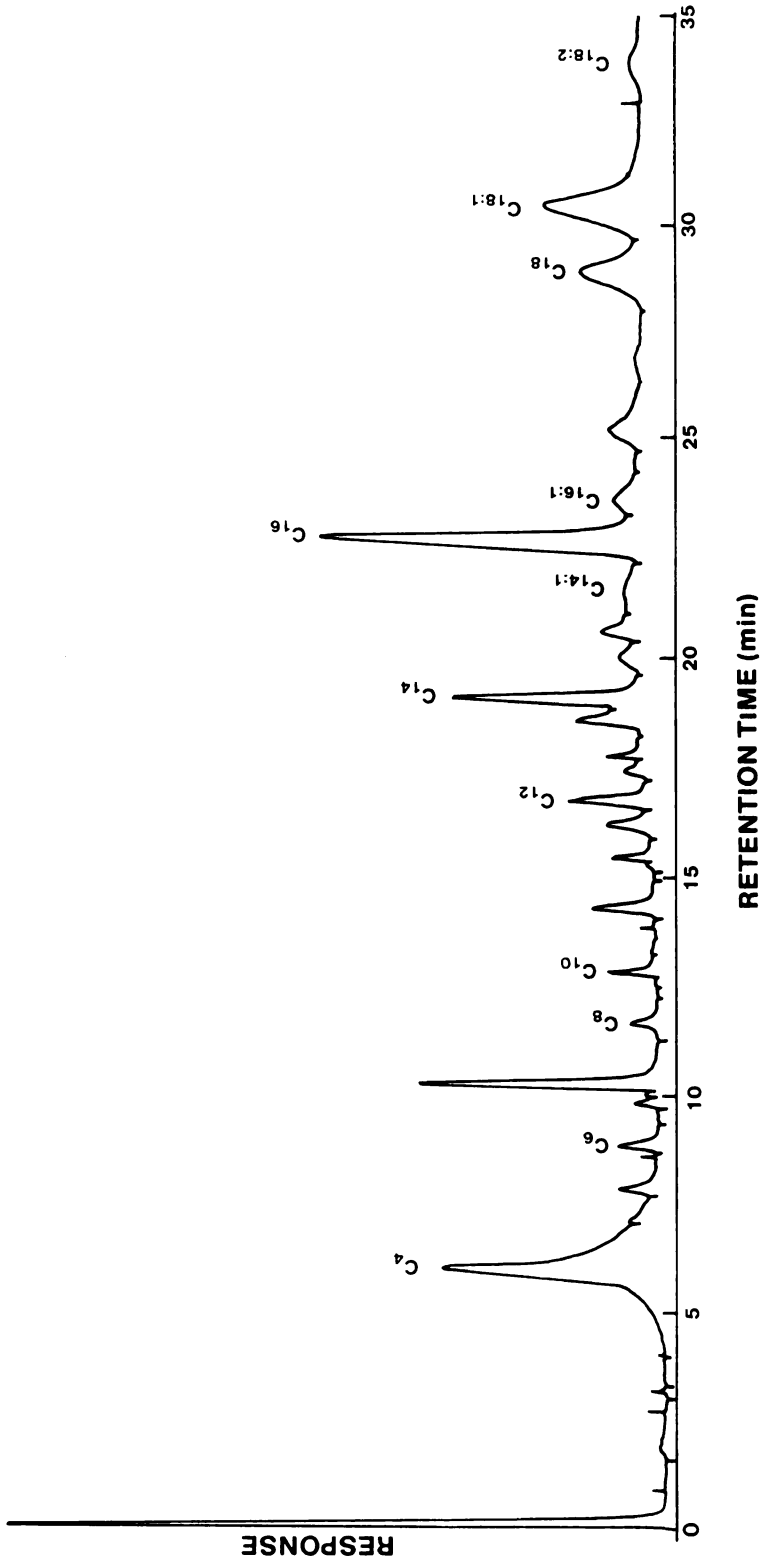

RESPONSE
0
5
10
15
20
25
30
35
RETENTION TIME (min)
C4
C6
C8
C10
C12
C14
C14:1
C16
C16:1
C18
C18:1
C18:2

Figure 24. Gas chromatogram of free fatty acid, in butter oil.

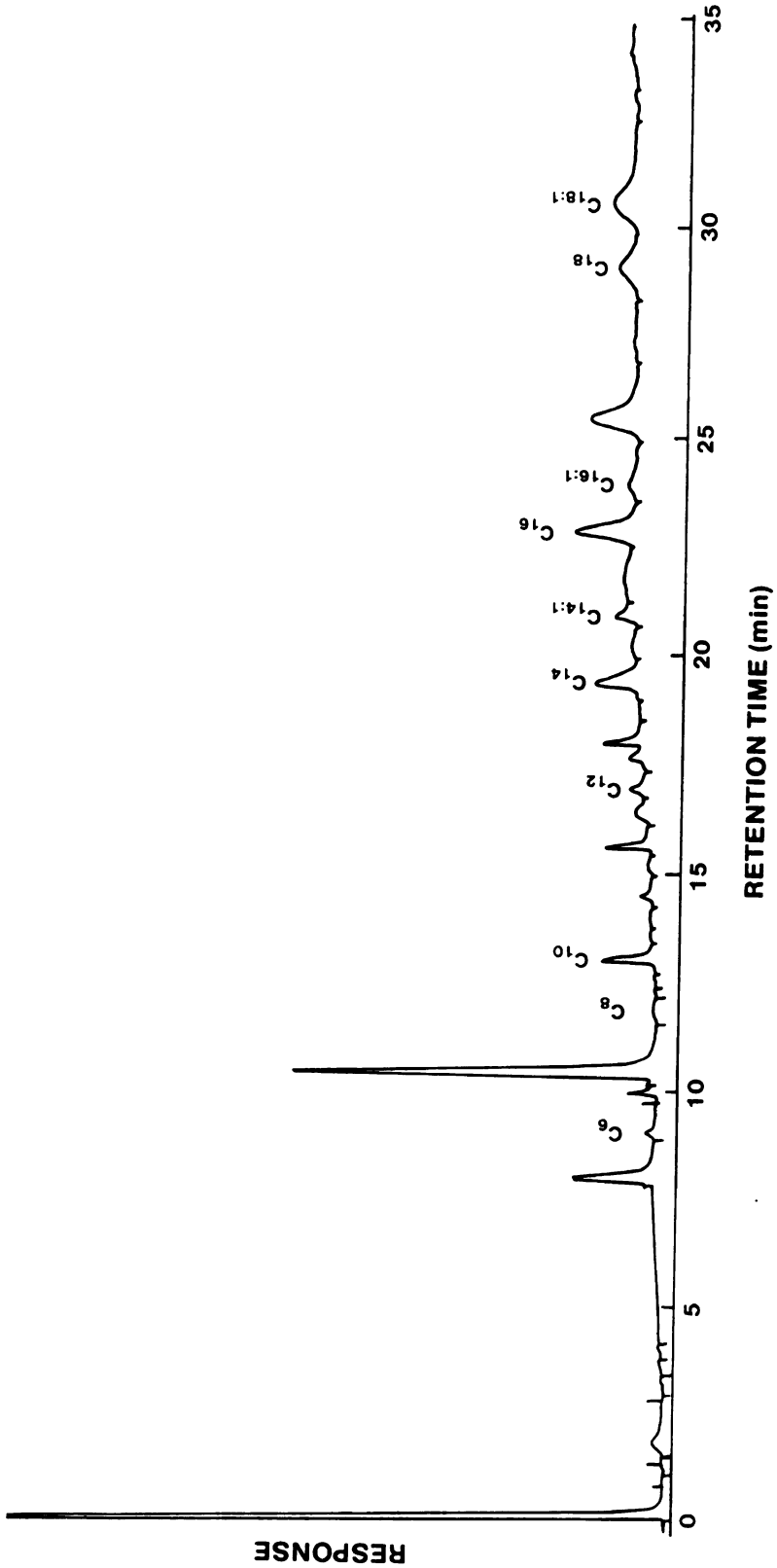
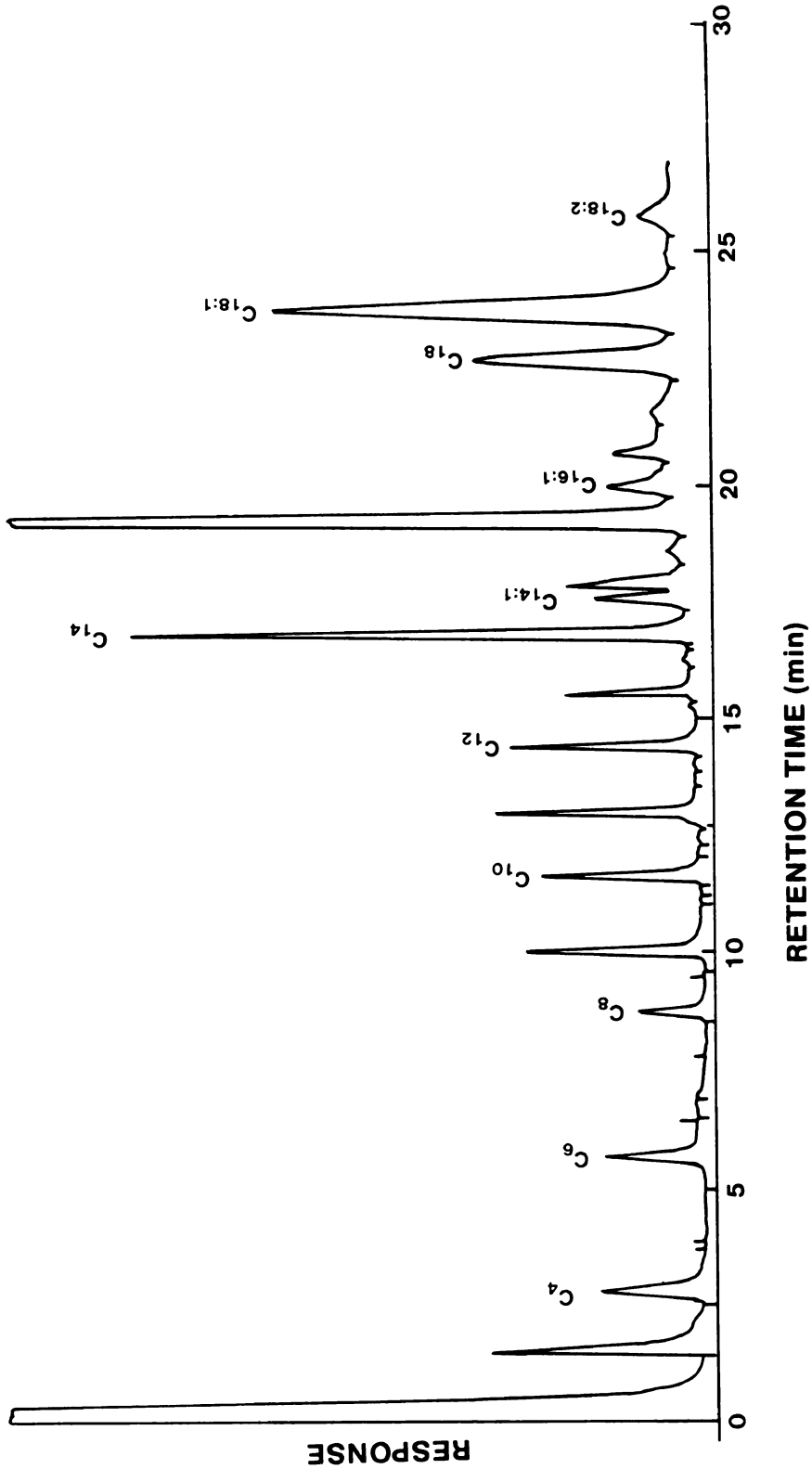

RESPONSE
0
5
10
15
20
25
30
35
RETENTION TIME (min)
C6
C8
C10
C12
C14
C14:1
C16
C16:1
C18
C18:1

Figure 25. Gas chromatogram of fatty acids composition of butter oil determined by transesterification.


RESPONSE
RETENTION TIME (min)
0
5
10
15
20
25
30
C4
C6
C8
C10
C12
C14
C14:1
C16
C16:1
C18
C18:1
C18:2

SUMMARY AND CONCLUSIONS

Growth and Lipase Production by *Penicillium caseicolum*

Penicillium caseicolum C_1 was grown and maintained in Czapek-Dox agar slant at 25 ± 1°C for 7 days. The fungal growth was suspended in 0.1 M phosphate buffer, pH 7.0, then inoculated into the media at rate 1%. The spore counts in the inoculum were 1.16×10^7 to 1.44×10^7/ml.

In order to determine the best growth conditions for *Penicillium caseicolum*, five fungal media were chosen. These media were: Czapek-Dox broth, Sabouraud Maltose broth, Sabouraud liquid medium, mycological broth and malt extract broth. The media were sterilized, inoculated with 1% spore and cell suspension and incubated at 23 ± 1°C under stationary conditions. The growth was harvested after 4, 7, 10 and 14 days, and evaluated by dry cell weight measurements. The cultures were filtered through Whatman filter paper #5, and washed twice with distilled water. The dry weight was determined after drying in a vacuum ovum (30 inch) at 100°C for 5 h. These conditions were established to achieve constant weight. The fungal growth in Czapek-Dox broth and mycological broth was higher, followed by Sabouraud maltose broth, Sabouraud Liquid medium and maltose extract broth. Czapek-Dox broth and mycological broth were chosen for

further studies.

During all stages of this investigation, lipase activity was determined by silica gel column chromatographic procedure or pH-stat method. Ten percent tributyrin or butter oil emulsified in 10% gum arabic solution containing 8 μM sodium taurocholate and 8 mM $CaCl_2$ was used as substrate.

Penicillium caseicolum growth and lipase production in Czapek-Dox broth and mycological broth at 25 ± 1°C under stationary conditions as well as under submerged (shaking) condition at 120 rpm were compared. Lipolytic activities were determined by silica gel method. The results indicated that Czapek-Dox broth and mycological broth supported better growth and lipase production under submerged (shaking) than stationary conditions.

Five strains were studied for their growth, spore count and lipase production in mycological broth containing 1% corn oil. The data showed there was little difference in the growth among various strains. Lipase activity varied widely and was highest with strain B_5. No relationship between growth, spore count and lipase activity was evident.

One percent corn oil, butter oil and olive oil were added to mycological broth and Czapek-Dox broth. In Czapek Dox broth, all the oils had inhibitory effect on lipase production. When mycological broth was used, corn oil, olive oil and butter oil had stimulatory effect on lipase production. Maximum activity was obtained after 4 days of

growth of Penicillium caseicolum. The lipase activity decreased after 7 days of growth. The lipase production was considerably higher with corn oil incorporation in the medium as compared to the olive oil and butter oil addition. Investigation on lipase activity of Penicillium caseicolum on daily basis in mycological broth showed that the highest lipase activity was obtained after 3 days of growth after which it declined. Using olive oil in the medium, the lipase activity was highest on the second and fifth day of growth. In the next study, addition of soytone into Czapek-Dox broth resulted in an increase of 61% in lipase production as compared to mycological broth after 4 days of fungal growth.

Different sources of nitrogen were used in the growth media of Penicillium caseicolum. The pH of the medium dropped between 0.57 and 2.08, depending on the source of nitrogen, reflecting the degree of amino acid uptake. Soytone was found to be the best source of nitrogen and showed slightly higher lipase activity than peptone. The lowest lipase activity was with 20% whey protein concentrate.

Replacing dextrose with different sources of carbon in the growth media of Penicillium caseicolum showed a decrease in pH by 1.2 to 1.94. The growth rate was highest with lactose followed by galactose, fructose, dextrose, sucrose and maltose. Dextrose showed highest lipase activity, followed

by sucrose, maltose, galactose, lactose and fructose. Lipase production in media containing dextrose and sucrose was comparable. Also the results showed no relationship between growth and lipase production with different sources of carbon.

The addition of certain minerals to mycological broth showed inhibitory effect on growth and lipase production to various degrees. The results indicated no relationship between growth and lipase production.

The growth and lipase production of Penicillium caseicolum was markedly affected by pH of the medium. Fungal growth generally increased with incubation period. The optimum pH for growth was 4.7. The highest lipase production was obtained after 3-4 days of growth. The optimum pH for lipase production was 7.0. A decrease in the lipase production was observed below or above pH 7.0.

The optimum growth and lipase production by Penicillium caseicolum was at 25^{o}C. Above this temperature, a sharp decrease in the lipase production was observed.

Lipase production increased with an increase in the degree of agitation of the medium. The lipase activity reached its maximum after 2 days of fungal growth at 500 rpm. The growth of the fungal was higher at 500 rpm than at 120 and 200 rpm.

Characterization of the Lipase

The optimum temperature for lipase activity of Penicillium caseicolum was 35°C, when butter oil and tributyrin were used as substrates.

The optimum pH of the enzyme was 9.0. There was a sharp decrease in lipase activity above the optimum pH for both the substrates. The lipase activity was 3-4 times higher with tributyrin as compared to butter oil substrate.

Addition of bile salts and calcium chloride to the substrate affected the reaction to varying degrees. When tributyrin was used as a substrate, bile salts and $CaCl_2$ individually or in mixed form had inhibitory effect. In case of butter oil, bile salts had stimulatory effect at 1-10 µM concentrations as compared with no salts. Calcium chloride stimulated the rate of hydrolysis at low concentrations (2 mM) and had an inhibitory effect at higher concentration. Stimulatory effect was highest with a mixture of 8 µM sodium taurocholate and 8 mM $CaCl_2$/ml with butter oil substrate.

Lipase from Penicillium caseicolum was stable at -26.6 and -15°C for one month. It was stable up to 3 days at 0°C and 4°C. The initial rate of hydrolysis decreased by 1, 58, and 87% on storage for 3 days at 25, 37 and 45°C, respectively. The enzyme was completely inactivated after 24 h of storage at 55°C. Upon pasteurization, boiling and autoclaving, the lipase was 98-100% inactivated. The Z_D

value was 15.8°C.

Penicillium caseicolum lipase showed specificity toward simple triglycerides containing short chain fatty acids, especially C_4. The rate of hydrolysis of tributyrin was 4 times higher than that of triolein. In case of natural lipids, lipase activity was higher toward lipids containing high polyunsaturated fatty acids. Corn oil and sunflower oil were hydrolyzed 1.5 times faster than butter oil.

Gas chromatographic analyses showed that the lipase was highly specific for the release of butyric acid from butter oil.

APPENDIX

Table A1. List of chemicals used in this study.

Chemical	Reference Number	Company
Acetone	2440	Mallinckrodt
Ammonium hydroxide	1177	Mallinckrodt
Ammonium sulfate	3512	Mallinckrodt
BF_3-Butanol (14% w/v)	3-3125	Supelco
BF_3-Methanol (14% w/v)	3-3020	Supelco
Bromocresol green	5-983-5	Fisher Scientific
Butyl Alcohol (normal)	2990	Mallinckrodt
Calcium caseinate		Liberty Enterprise
Calcium chloride	1-1332	Baker
Calcium sulfate	4300	Mallinckrodt
Casein	C-203	Fisher Scientific
Casamino acid	B-230	Difco
Chloroform	4440	Mallinckrodt
Dextrose	4912	Mallinckrodt
Dipotassium phosphate	7092	Mallinckrodt
Ethanol		Aaper Alcohol and Chemical
Ethyl ether	0844	Mallinckrodt
Ethyl glycol	5001	Fisher Scientific
Ferrous sulfate	2070	Baker
Fructose	L-95	Fisher Scientific
Galactose	B163	Difco
Gum Arabic	G9752	Sigma

Table A1. (cont'd).

Gamma globulin		BioRad Laboratories
Hexane	5189	Mallinckrodt
Hydrochloric acid	2612	Mallinckrodt
Iso-propyl alcohol	5-9084	Baker
Lactic acid	1-0194	Baker
Lactose	B-156	Difco
Magnesium sulfate	1-2506	Baker
Maltose	B-169	Difco
Methanol	3024	Mallinckrodt
Neutralizing agent	3-3052	Supelco
Phenolptalein		Mallinckrodt
Phenol red		United States Biochemical Corp.
Phosphoric acid	2788	Mallinckrodt
Potassium acid phtalate	6704	Mallinckrodt
Potassium chloride	6858	Mallinckrodt
Potassium hydroxide	6984	Mallinckrodt
Proteose peptone	B-120	Difco
Silicilic acid	2847	Mallinckrodt
Sodium caseinate		Liberty Enterprise
Sodium chloride	7581	Mallinckrodt
Sodium dibasic phosphate	3824	Baker
Sodium desoxycholate	B-248	Difco
Sodium hydroxide	7708	Mallinckrodt
Sodium monobasic phosphate	7892	Mallinckrodt

Table A1. (cont'd).

Sodium nitrate	1-3780	Baker
Sodium taurocholate	S07270	Pfaltz & Bauer
Sucrose	1-4072	Baker
Sulfuric acid	2468	Mallinckrodt
20, 29% whey protein		Sheffi Ltd.

Table A2. List of microbiological media used in this study.

Media	Reference number	Company
Agar	B-140	Difco, Detroit, MI
Czapek-Dox broth	B-338	" "
Sabouraud Liquid medium	B-109	" "
Malt extract broth	B-113	" "
Peptone	B-118	" "
Mycological broth	B-406	" "
Sabouraud Maltose broth	B-429	" "
Soytone	B-436	" "
Trypticase	02-148	BBL, Cockeysville, MD

Table A3. List of natural & and synthetic lipids used in this study.

Lipid	Purity %	Reference number	Company
Tributyrin	96-98	T4637	Sigma Chemical Co.
Tricaproin	90+	T4137	Sigma Chemical Co.
Tricaprylin	90+	T9126	Sigma Chemical Co.
Trilaurin	90+	T3127	Sigma Chemical Co.
Trimyristin	90+	T7252	Sigma Chemical Co.
Tristearin	90+	T6628	Sigma Chemical Co.
Triolein	75+	T7752	Sigma Chemical Co.
Tripalmitin	90+	T8127	Sigma Chemical Co.
Almond oil			Etsguenard-France
Butter oil			Made from Land O' Lakes butter
Corn oil			Best Foods, CPC International Inc., NJ
Grapeseed oil			Soleillou-France
Hazelnut oil			G. Viver-France
Lard			Armour, Phoenix, AZ
Olive oil			Pompeian, Baltimore, MD
Peanut oil			Hunza, distributed by M and J Foods, L.A., CA
Sesame oil			Hunza, "
Soy oil			Hunza, "
Safflower oil			Hunza, "

Table A3. (cont'd).

Sunflower oil	Hunza, distributed by M and J Foods, L.A., CA
Walnut oil	Rougre-France

*Commercial oil no purity.

Table A4. List of free fatty acids standard* used in this study.

Fatty acid	Reference number	Company
Butyric acid	FA0040	Alltech Association
Caproic acid	FA0060	" "
Enanthic acid	FA0070	" "
Caprylic acid	FA0080	" "
Capric acid	FA0100	" "
Lauric acid	FA0120	" "
Myristic acid	FA0140	" "
Myristoleic acid	M6129	Sigma
Palmitic acid	FA0160	Alltech Association
Palmitoleic acid	P0875	Sigma
Margaric acid	FA0170	Alltech Association
Stearic acid	FA0180	Alltech Association
Oleic acid	FA0181C	" "
Linoleic acid	FA0182C	" "

*Purity 99+%.

Table A5. List of instruments used in this study.

Instrument	Company
Autoclave (Type 20)	Wilmot Castle Company, Rochester, NY
Balance Mettler H30	Mettler Instrument Corporation, Highstown, NJ
Balance (Mettler top load Type 120)	Mettler Instrument Corporation, Highstown, NJ
Centrifuge RC2b (Automatic refrigerator)	Sorvall, Newtown, CT
Digestor MicroKjeldahl FF699	Lab. Con Co., Kansas City, MO
Distillator (Microkjeldahl)	Fisher Scientific, Pittsburg, PA
Fermentor (Microform) MF107	New Brunswick Scientific Co., New Brunswick, NJ
Gas chromatography Model 5840A	Hewlett Packard, Avondale, PA
Haemocytometer (improved Neubauer 2936-C10)	Thomas and Arthur Co.
Hand Homogenizer (11-504-200)	Fisher Scientific, Pittsburg, PA
Incubator Model 4 (3148D)	Precision Scientific Co., Chicago, IL
Incubator Blue M (Model 200A)	Blue M Electric, Blue Island, IL
pH Controller Model 43DPHCR	Virtis, Gardiner, NY
pH-electrode (Autoclavable) (XPHD-700)	Ingold Electrode Inc., Andover, MA
pH-Meter CHEMTRIX60A	Chemtrix Inc., Killboro, OR
pH-Pump Model 43pH-PE	Virtis Co., Gardiner, NY
pH-Stat Titrator-E526	Metrohm, Herisau, Switzerland
Motor Drive Piston Burette E-525	Metrohm, Herisau, Switzerland

Table A5. (cont'd.).

Potentiometer Recorder Servagor 210	Metrohm, Herisau, Switzerland
Constant temperature circulator Model 80	Fisher Scientific, Pittsburg, PA
Shaker Model G-25	New Brunswick Scientific Co., New Brunswick, NJ
Spectrophotometer-20	Bausch and Lomb, Rochester, NY
Vacuum Oven Model 19	GCA/Precision Scientific Co., IL
Vacuum Pump Welch (Model 1402)	Sargent Welch Scientific Co., IL

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