QUANTITATION OF SOIL LIPASE

WITH 4-MUB FLUORESCENCE

By

SUNIL KUMAR PANCHOLY

Bachelor of Science
University of Udaipur
Udaipur, Rajasthan, India
1964

Master of Science
University of Udaipur
Udaipur, Rajasthan, India
1966

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1968

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of the Oklahoma State University
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Thesis Approved:

J. C. Lynd
Thesis Adviser

Ralph S. Matlock

Lester W. Reed

George V. Odell

D. D. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Microbiological interactions, metabolic pathways, and biochemical controls of plant-animal lipid transformations to soil humic components are not known. However, decomposition and synthesis of fats, waxes, oils, resins, and steroid compounds within soils are of particular concern at the present time for pesticide degradation, pollution controls, and induced accelerated dissimilation of recalcitrant organic compounds.

Diversity in composition of plant and animal materials presents to soil microflora substrates with a wide spectra of lipid substances that are physically and chemically heterogeneous. Most require biological transformations catalyzed by extracellular enzymes outside the microorganism. Eventually these enzymes hydrolyze lipid macromolecules to moieties or degradation products that may be absorbed as nutrients and resynthesized in microbial metabolism.

Soil microbiological research for systematic determinations of lipid transformations have been limited due to precision deficiencies of the available lipase quantitation procedures. Recent developments with improved instrumentation and techniques present good possibilities for elucidation of governing factors in lipid transmutations within soil biological systems.

The objective of this study was to evaluate governing parameters

of lipase activity in soil biological systems with differential fluoresence using 4-Methyl Umbelliferone Butyrate.

CHAPTER II

LITERATURE REVIEW

Soil organic matter constituents are continually altered to other chemical forms, usually more simple organic and inorganic compounds, through enzymatic activity of soil microorganisms. The breakdown of complex organic residues within soil is primarily a hydrolytic process made possible by extracellular enzymes excreted by the decomposing microorganisms into the soil medium. Recent reviews by Skujins (24) and Porter (21) have summarized the limited literature published to date concerning research with soil enzyme systems.

In considering the dynamic interrelated biochemical activities in soil, Quastel (24) proposed a concept of considering soil as a biological entity, i.e. as a "tissue". Attempts at isolating enzymes in pure form from this complex soil matrix "tissue" have been unsuccessful. These enzyme studies were greatly affected by the strong binding of proteins by clays and humus components.

Alexander (1) noted the great variation in the materials added to soil including plant and animal materials, manures, fertilizers and various pesticides. This diversity of materials presents to the microflora a variety of substrate substances which are both physically and chemically heterogeneous. Herbicides and pesticides contain large amounts of carrier materials whose reactions in soils are completely unknown. Emulsions used in pesticide formulations to

control insects, pests and weeds contain large amounts of fatlike material and their fate in soil is yet to be determined.

Apparently, soil microbial transformation of plant materials, such as proteins, fats and certain other organic substances, is of a similar nature as with lignin and cellulose. Biopathways and eventual fate of plant residues during humification can be that:

(1) They are decomposed completely.

(2) The decomposition products participate as components in the formation of a molecule of humic substance.

(3) The plant substances participate in the formation of humic substances through products of bacterial resynthesis.

Thus plant and animal components can be direct or an indirect source of humic substances (1).

In 1925, Winogradsky (30) put forward his concept that organisms which decompose fresh organic matter in soil belong to an ecologically separate flora from those which decompose humus. These he termed "autochthonous" flora. "Zymogenous" organisms only developed when soil was enriched with the particular substrate.

Waksman (30) has shown that fractions of soil organic constituents are ether and alcohol soluble. These lipid fractions contain fats, oils, waxes, resins and a number of pigments. As such, this fraction is resistant to decomposition because of its conjugation with other constituents. Fats and oils, when acted upon by specific enzymes, eventually breakdown to fatty acids, glycerol, esters and other simple compounds which can again be readily utilized (13). Hydrolysis of triglycerides and other fat materials is catalysed by lipases. Pokorna (20) has determined lipase activity in high moisture bog soils by

incubating with an olive oil emulsion substrate and then titrating the fatty acids with standardized sodium hydroxide. This proposed method for determining soil lipolytic activity is basically as follows:

$$\text{Triglyceride} + 3H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

This method apparently was satisfactory with peat and muck soils but was not reliable with mineral soils where there are low levels of enzyme activity and many interferences with H^+ titrations.

Enzyme studies in soils have been difficult. Porter (21) mentioned that it has not been possible to isolate enzymes from soils because of their unknown origin, distribution and localization. According to McLaren (17) principal methodological problems with the enzymatic studies have been to achieve an effective inhibition of microbial activity and at the same time leave the soil enzymes unaffected. He introduced electron beam sterilization of soils to accomplish this. Another problem is the adsorption of enzymes on the soil clay fractions. Mortland (18) determined the influence of clay minerals on enzymatic hydrolysis of organic phosphate compounds.

Urease activity and urea transformations in soils have been of much interest to soil and plant scientists. Conrad (5) studied urease activity at various soil depths. Stojanovic (27) from his studies in hydrolysis of urea in soil as affected by season and added urease, concluded that urease-like activity of soil depends primarily on type of soil, reaction, depth of profile, temperature and cultural practices. Under normal conditions the hydrolysis of urea is largely brought about by the action of a number of soil microorganisms. He concluded that those field conditions affecting the size and activity of the microbial population of a specific region would influence the amount of urease

produced and thus the capacity of the soil of that region to hydrolyze urea.

Jackman and Black (9) concluded from their study that phytase activity was closely correlated with microbial metabolism. Sorensen (22) reported that xylanse activity was increased by a factor of six or more when straw was applied to the soil. Troller and Bozeman (29) isolated, purified and characterized lipase extracted from a Staphylococcus strain and observed that the optimal activity of the purified lipase was at pH 7.5.

Changes in soil environmental factors, both agronomic and climatic, that would be expected to influence the activities of microorganisms, also influence the magnitude of many enzymatic reactions (3, 11, 14). Soil enzymes, just as those of plants and animals systems, are specific in nature and are greatly affected by pH, temperature, soil aeration, moisture, and addition of various amendments as well as soil organic matter (12, 14, 26).

Soil lipases are an important class of enzymes from the point of view that they are mainly responsible for the transformations of all fat like material in soil and lipid components. However, no reliable method is now available for soil lipase quantitations (22).

Guilbault, et al. (7, 8) and Jacks and Kircher (10) have described a simple, rapid, and accurate procedure for the assay of lipase activity in the presence of other esterases based on the hydrolysis of 4-Methyl Umbelliferone Butyrate catalyzed by lipase as follows:

$$\text{4-MUB (nonfluorescent)} \xrightarrow{\text{lipase}} \text{4-MU (fluorescent)}$$

The hydrolyzed product, 4-Methyl Umbelliferone is highly fluorescent and can be detected at very low concentrations by use of a

fluorescence spectrophotometer. This study was undertaken to evaluate governing parameters of lipase activity in soil biological systems with differential fluorescence using 4-Methyl Umbelliferone Butyrate.

CHAPTER III

MATERIALS AND METHODS

Reference enzymatic studies were conducted with wheat germ lipase, per Singer (23), with a minimum activity of 0.1 IU/mg (Nutritional Biochem. Co.). The enzyme assay utilizing induced 4-MUB fluorescence was as follows:

(1) 3.0 ml of 0.1M phosphate buffer, pH 7.5. Buffer was prepared by using NaH_2PO_4 and then adjusting the pH to 7.5 with NaOH.

(2) 0.1 ml of enzyme solution 1 mg/ml.

(3) 0.1 ml of 10^{-2}M 4-MUB prepared in ethylene glycol monoethyl ether.

The fluorescence of the solutions was determined with a Perkin-Elmer 203 Spectrophotofluorometer at λ excited 340 mμ and λ emitted 450 mμ between cross polarizing lens. The instrument was initially adjusted to zero for each determination and then read for three minutes at the controlled room temperature, as suggested by Guilbault, et al. (7).

These soil enzyme studies were conducted with a Psammentic Paleustalf, Eufaula sand. The physical and chemical analysis of this soil is presented in another publication (19). Proximate analysis was 90% sand, 7% silt, 3% clay, 0.5% organic matter, exchange capacity of 2.7 meq/100 g and pH 6.7.

Two methods were used for determination of soil lipase activity.

(1) Fatty acid titration: To 10g of soil, 3 ml of toluene was

added to stop microbial activity. After 15 minutes, 4 ml of distilled water and 3 ml of olive oil substrate were added. (Olive oil substrate: 165 ml 10% gum acacia, 15 grams crushed ice, and 20 ml of olive oil, U.S.P. adjusted to pH 8.0). Soil was incubated for 72 hours at 30°C in a temperature controlled shaker. After incubation, the soil was extracted with 10 ml of alcohol-ether 1:1 (V/V) solution for 20 minutes. Ten ml of this extraction was then titrated against 0.05N NaOH using thymolphthalein as the indicator. Amount of fatty acids present in the extract were then calculated as milliequivalents. Substraction of check soil titration values yielded apparent soil lipase activity. This procedure was a modification of Pokorna's method (20).

(2) Induced MUB fluorescence: 10 g of soil was extracted with 20 ml of 0.1 M phosphate buffer, pH 7.5. The mixture was shaken for 30 minutes at 30°C, then filtered through Whatmann No. 1 filter paper. To 3.1 ml of this mixture, 0.1 ml of 1 X 10^{-2} M 4-MUB was added. This assay mixture was mixed thoroughly and fluorescence determined for 3 minutes at λ excited 340 and λ emitted 450 mμ between cross polarizing lens.

Protein determination for both wheat germ lipase and soil extracts utilized the Folin Ciocalteu procedure (16).

Reagent A	2% Na_2CO_3 in 0.1N NaOH
Reagent B	2% Sodium tartrate
Reagent C	1% $CuSO_4.5H_2O$
Reagent D	Mixed 50 ml of reagent A with 0.5 ml reagent B and 0.5 ml of reagent C
Reagent E	Folin reagent diluted to 1.0 N

To the sample in 1 ml water, add 5 ml of reagent D followed by

mixing. The solution was allowed to stand at room temperature, 23° C±1, for 10 minutes then 0.5 ml of reagent E was added and mixed immediately. The blue color produced was read after 30 minutes at 725 mμ on a B & L Spec-20 Spectrophotometer. A standard curve for lipase protein was also determined using wheat germ lipase.

Characterization of Wheat Germ Lipase with 4-MUB Fluorescence

Wheat germ lipase was used to establish the linear response between induced fluoresence and enzyme concentration. Five different enzyme levels were used: 3.300×10^{-3}, 1.650×10^{-3}, 0.825×10^{-3}, 0.413×10^{-3} and 0.206×10^{-3} units/ml of assay volume. Substrate 4-MUB was kept constant at 1.0×10^{-2} M.

Effect of substrate concentration was determined by using five different levels of 4-MUB, 1×10^{-2}M, 5×10^{-3}M, 2.5×10^{-3}M, 1.25×10^{-3}M and 6.25×10^{-4}M with concentration of wheat germ lipase constant at 3.300×10^{-3} units/ml of assay volume.

Vmax and Km of the reaction were determined by calculation with both the Michaelis plot and the Lineweaver-Burk method.

Enzymes are active over a limited pH range and effects of four pH levels 6.5, 7.0, 7.5, and 8.0 were determined in terms of induced MUB fluorescence with wheat germ lipase.

The effects of differential temperature on the velocity of the enzyme-MUB reaction were determined by using three temperature levels, 5°C, 30°C and 80°C.

Effects of various cations and anions in solution on lipase-4 MUB reaction were determined with acetate solutions for: Zn^{++}, Pb^{++}, Cu^{++}, Hg^{+}, Ag^{+}, Al^{+++}, Fe^{++}, Fe^{+++}, Co^{++}, and Mn^{++} at concentration of

1×10^{-1}M.

Ca^{++}, Mg^{++}, K^{+} and Na^{+} acetates were also used at the following levels: 1×10^{-1}M, 1×10^{-2}M, 1×10^{-3}M, and 1×10^{-4}M, 1×10^{-5}M in assay mixture.

Various combination of these cations were used after the optimum levels of individual cations were established. The assay salt solution were mixed thoroughly with 5 minutes reaction time before MUB was added and fluorescence determined.

Selected anions were also screened in combination with the cations that did affect the enzymatic reaction. These anions included: citrate, cyanide, carbonate, sulfite, hydroxide, nitrate, nitrite, chloride, sulfate and bicarbonate at concentrations of 1×10^{-1}M.

Effect of various inhibitors on lipase-4 MUB reaction were determined with percent fluorescence inhibition as follows:

$$\frac{\text{check (no inhibitor)} - \text{inhibitor}}{\text{check (no inhibitor)}} \times 10^{2} = \% \text{ inhibition}$$

Various inhibitors used included aflatoxin, cupric acetate, ferric chloride, sodium cyanide, sodium sulfide and ethylene-diamine tetra acetic acid. All inhibitors were used at 0.1M concentration with 5 minute reaction times before 4-MUB added and fluorescence was determined. Fluorescence visualization with long wave UV light (360-366 mμ) was done with a 15 X 5 cm strip of glass fiber filter paper No. 934-AH (Hulburt Paper Co.) soaked thoroughly with 8.0×10^{-5}M MUB in sodium phosphate buffer and placed on a thin glass plate. Wheat germ lipase at levels of 5×10^{-3}, 2.5×10^{-3}, 1.25×10^{-3}, 0.63×10^{-3} and 0.31×10^{-3} units as 5 μl spots were applied on the strip. After 3 to 5 minutes, the fluorescence was recorded within a model C-6 Chromata-Vue cabinet with contrast filter and Transilluminator

(Ultra Violet Products Inc., San Gabriel, California) at 360-366 mμ for fluorescence emission determinations. An MP-3 Industrial View Polaroid Camera was used with a hand made cone adapter for the fluorescent viewer cabinet. Polaroid film types used in these studies included black and white series 107 (ASA 3000) and color film 108 (ASA 75).

Determination of Soil Lipase Activity with 4-MUB Fluorescence

The procedures for determining soil lipase activity by free fatty acid titration and by 4-MUB fluorescence has been described earlier in this chapter. Soil cultures which received 2% olive oil and incubated 15 days at 30°C were used in the initial studies. The soil extract used was equivalent to 0.25, 0.5, 1.00, 2.00 and 3.00 g of soil. The volume of phosphate buffer for assay was 3.0 ml in each case. Fluorescence was determined after addition of 4-MUB at 1.0 X 10^{-2}M. Two similar studies were set up in order to confirm induced soil enzymatic activity. The soil enzyme activity was completely destroyed in these studies with 10% trichloroacetic acid or by heating at 80°C for 5 minutes.

Preliminary studies were conducted to determine the effects of olive oil addition on soil lipase activity. Eufaula soil was amended with 1, 2, 3, 4, and 5 percent olive oil and these cultures were incubated 15 days at 30°C. At the end of this incubation period, extractions were made and MUB fluorescence determined. Protein determinations were also made on the soil extracts.

In another series of studies the effect of incubation time was determined with the addition of 1 and 2% olive oil and 1, 2, and 4 percent peanut residue. Composition of the peanut residue was as

follows: 1.89%N, 0.11%P, 0.50%K, 0.34%Ca. 6.28% fat and 18.4% fiber. Days of incubation were 5, 10, 15, 20, 25, and 30.

Effects of various levels in soil solution on soil lipase activity were determined with Ca, Mg, Na and K as acetate salts added to the nontreated soil as well as the soil which received 4% olive oil. Cation levels were 0.1, 0.2, and 0.4 M/10g soil and all cultures incubated for 15 days at 30°C. At the end of the incubation, lipase activity of these cultures was determined along with electrical conductivity and soil pH.

Effects of various inhibitors on soil lipase activity were determined for the inhibitors active with the wheat germ lipase studies. EDTA, Fe^{+++}, Cu^{--}, and S^{--}, were used at 1 X 10^{-1}M and 1 X 10^{-5}M levels. Soil extracts were used from soil enriched with 4% olive oil and incubated for 15 days at 30°C. The 4-MUB substrate concentrations were 1 X 10^{-2}M, 5 X 10^{-3}M, 2.5 X 10^{-2}M and 1.25 X 10^{-3}M. The inhibitor solution was added to the soil extract and mixed with 5 minutes reaction time before 4-MUB was added and activity determined.

Effects of plant oils with contrasting fatty acid composition on soil lipase activity were determined at three different soil pH levels, 4.8, 7.7, 8.6. Composition of the oils were as follows:

	% Saturated Fatty Acids			% Unsaturated Fatty Acids		
	Total	Palmitic C_{16}	Stearic C_{18}	Oleic C_{18-2H}	Linoleic C_{18-4H}	Other Unsaturated
Peanut Oil	19	8	6	50	31	-
Coconut Oil	91	10	2	7	1	1
Safflower Oil	8	3	4	15	76	1
Corn Oil	12	8	3	30	55	3
Olive Oil	12	9	2	80	8	-

These oils were added to the soil at 1% and 2% level, thoroughly mixed, and then incubated for 15 days at 30°C. After the incubation period, lipase activity was determined with MUB fluoresence.

Soil Microfloral Lipase Detection

Soil microfloral lipase detection with 4-MUB was accomplished with a large number of sterile glass Raschig rings (Mercer Glass Works, Inc.) having 0.60g soil capacity buried in cultures of untreated Eufaula and in soil cultures with 4% olive oil amendment. The soil cultures were incubated at 30°C for 15 days in a saturated atmosphere (Stults seed germinator model S-30, Stults Scientific Engineering Corp., Springfield, Illinois). Most probable numbers of soil microbial groups and individual species were obtained by extracting soil within single Raschig rings with 10 ml sterile distilled water and using sterile pipettes for dilutions in sterile water to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. One ml samples of these extract dilutions were then transferred with sterile pipettes to sterile petri dishes, agar media added, and the cultures then incubated at 30°C. Microbial counts and extra-cellular lipase were determined with 4-MUB after 72 hours incubation.

Three different media were used to determine the relative numbers and kinds of various microorganisms. For growth of bacteria, bacto-nutrient broth (Difco Laboratories, Detroit, Michigan) with composition /liter: beef extract 3.0 g, peptone 5.0 g and agar 8.0 g with final pH 6.8 was used.

Similarly actinomycetes isolation agar (Difco Laboratories) with composition/liter: sodium caseinate 2.0 g, asparagine 0.1 g, sodium propionate 4.0 g, dipotassium phosphate 0.5 g, magnesium sulfate 0.1 g,

ferrous sulfate 0.001 g and agar 15.00 g with final pH of 8.1 was used at 22 g per liter. After heating the medium to boil, 5 grams of glycerol was added before autoclaving at 15 psi for 15 minutes.

Soil inhabiting fungi were developed with potato dextrose agar, (Difco Laboratories). Composition of the potato dextrose agar per liter was: infusions from potatoes 200 g, dextrose 20 g, agar 15 g with a final pH of 5.6. To rehydrate the medium 39.0 grams of potato dextrose agar was dissolved in 1000 ml cold distilled water, then it was heated to boiling temperature and later autoclaved.

All plates were incubated at 30^{o}C for 72 hours. Plates were examined after 72 hours and bacterial colony counts taken. Some of the distinctive and representative plates were chosen to visualize the extracellular lipase activity. A 7 cm round glass fiber filter paper, No. 934-AH (Hulburt Paper Co.) was placed on the top of the medium in the petri dish. This filter paper was then soaked with 8.0×10^{-5}M 4-MUB and viewed with UV light. Recordings were made similarly as in experiment I (h) concerning wheat germ lipase.

CHAPTER IV

RESULTS AND DISCUSSION

Lipase activity with hydrolytic splitting of triglycerides to glycerol and fatty acids, illustrated in Figure 1, is determined with difficulty in biological systems (17). Limitations for fatty acid and glycerol determination are well recognized (20). The highly sensitive fluorometric determinations for lipase cleaving of the nonfluorescent butyryl ester of 7-hydroxy-4-methylcoumarin (4-MUB) to fluorescent 4-methyl umbelliferone, Figure 2, presents possibilities for soil lipid transformation studies.

Preliminary studies established similar mode of action by wheat germ lipase per Singer (23) as compared to apparent soil lipase reactions. Wheat germ lipase was used to evaluate the 4-MUB procedures for enzymatic soil lipid transformations. The "range finding" test was applied to find the optimal enzyme concentration for the assay where reaction rates were used to measure enzyme concentrations, Figure 3. This "valid range" of enzyme assay for wheat germ lipase at a concentration of 3.30×10^{-3} units/ml of assay volume was found to be optimal, Table 1, and later on used throughout this study. The increase of induced 4-MUB fluorescence at 1.0×10^{-2}M increased linearly with increases of lipase from 0.206 to 3.300 units/ml with $f(x) = 8.018$ and highly significant correlation coefficient and treatment F values.

Classical enzyme kinetic quantitations requires evaluation of

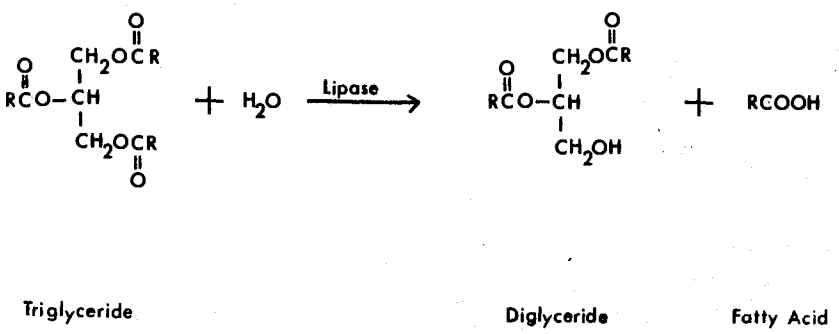
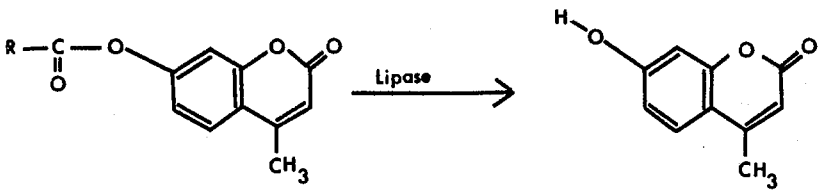


Figure 1. Initial hydrolysis of triglycerides with soil lipase to diglycerides and fatty acids.



4-MUB

Nonfluorescent

4-MU

Highly fluorescent

Figure 2. Nonfluorescent 4-Methyl umbelliferone butyrate hydrolysed by soil lipase to highly fluorescent 4-Methyl umbelliferone.

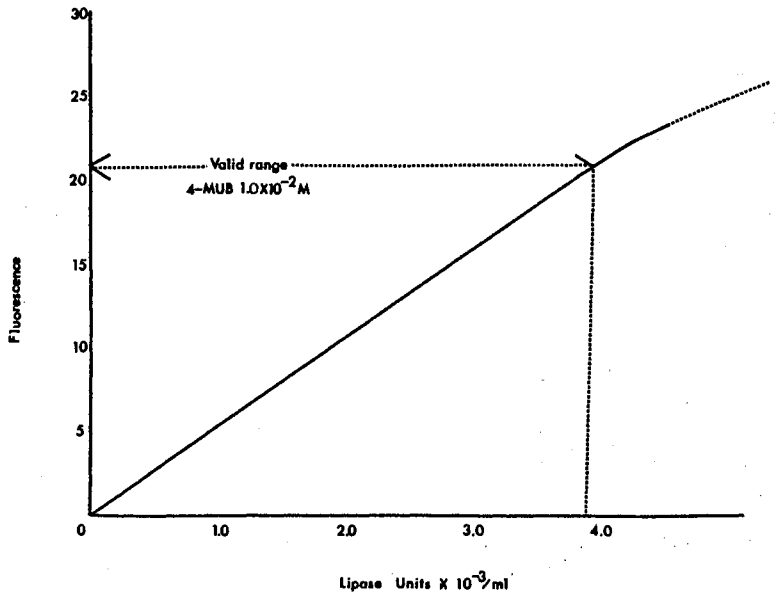


Figure 3. Wheat germ lipase (Singer) activity with induced 4-MUB fluorescence at optimal enzyme assay concentrations.

TABLE I

4-MUB FLUORESCENCE INDUCED WITH DIFFERENTIAL WHEAT GERM LIPASE LEVELS

Lipase Level Units X 10^{-3}/ml	Fluorescence I	II	III	Ave.
3.300	26	26	25	25.7
1.650	12	11	11	11.3
0.825	6	5	6	5.7
0.413	2	2	2	2.0
0.206	1	1	1	1.0

4-MUB level 1.0 X 10^{-2}M, r = .9986, f(x) = 8.018

Treatment F: 1,223 (P<.01), EMS .183

substrate concentration effects on enzyme reaction velocity. Results with 4-MUB concentrations varying from 6.25×10^{-4} to 1×10^{-2}M with constant lipase level of 3.3×10^{-3} units/ml are shown in Table II. Treatment F and correlation coefficient were highly significant yielding sound basis for calculations of Km = 3.1×10^{-5}M. When initial velocity was plotted against substrate concentration, a section of rectangular hyperbola was obtained as shown in Figure 4. The rate of wheat germ lipase catalyzed reaction increased with increasing substrate until a concentration of 1×10^{-2}M of 4-MUB was reached, beyond which further additions gave no increase. At this substrate concentration it was assumed that all the catalytically active sites of enzyme wheat germ lipase were occupied by the substrate. The enzyme concentration at this point was 3.30×10^{-3} units/ml of assay volume as previously determined as the optimum enzyme level.

Vmax and Km were determined by Michaelis plot in Figure 4 and Lineweaver-Burk plot in Figure 5. Vmax and Km of the wheat germ lipase (3.300×10^{-3} units/ml) and 4-MUB (1.0×10^{-2}M) reaction were 36.3 fluorescence units/3 minutes and 3.10×10^{-5}M, respectively (Figure 5). Guilbault, et al. (7) while comparing various substrates for lipase reported a lowest detectable concentration of lipase with 4-MUB as 4.00×10^{-4} mg/ml. Km values obtained by these workers for fluorescein dibutyrate, N-methyl indoxyl butyrate, 4-methyl umbelliferone heptanoate and 4-methyl umbelliferone octanoate were respectively, 7×10^{-6}, 2.9×10^{-5}, 7.3×10^{-6}, and 8.0×10^{-6}. The above values were obtained using porcine pancreas lipase with an activity of 0.2 units per mg of protein.

Enzymes are greatly influenced by pH levels and are only active

TABLE II

FLUORESCENCE OF 4-MUB AT DIFFERENTIAL CONCENTRATIONS INDUCED WITH WHEAT GERM LIPASE

Level moles/liter 4-MUB	Fluorescence I	II	III	Ave.
1×10^{-2}	37	36	36	36.3
5×10^{-3}	37	35	35	35.7
2.5×10^{-3}	33	33	34	33.3
1.25×10^{-3}	20	20	20	20.0
6.25×10^{-4}	13	13	13	13.0

Lipase level 3.3×10^{-3} units/ml, Km = 3.10×10^{-5}M, r = 0.76

Treatment F: 870:87 ($P<.01$), EMS .383

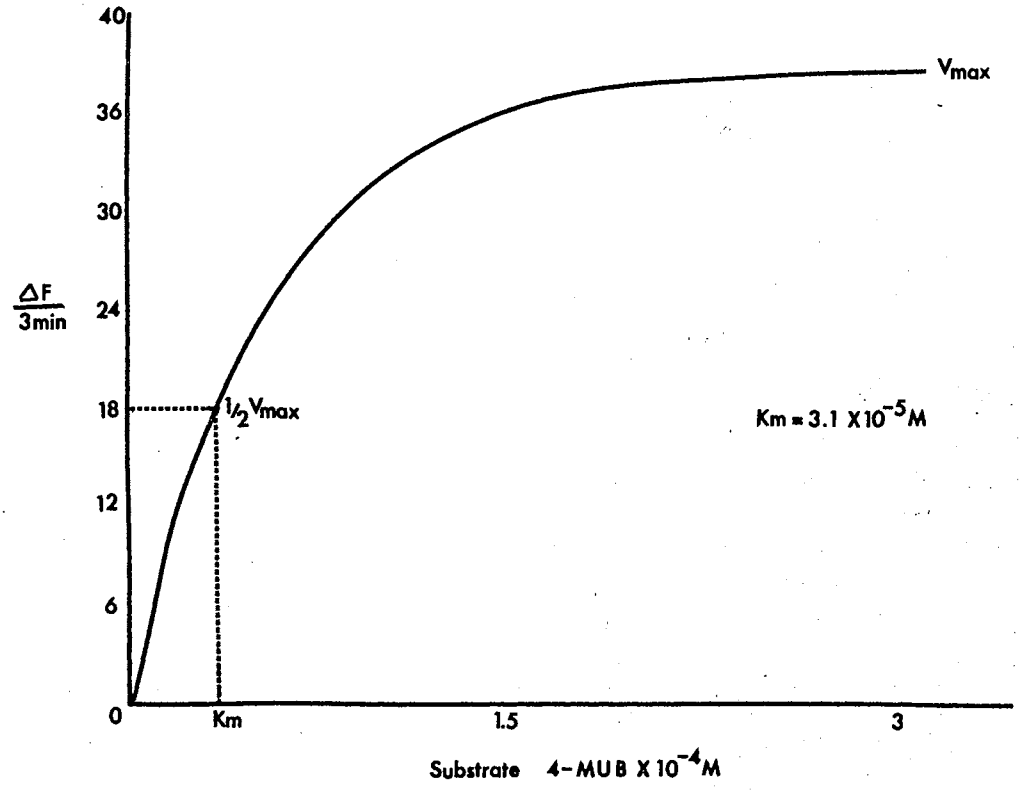


Figure 4. Wheat germ lipase activity with increasing 4-MUB substrate concentrations (Michaelis plot).

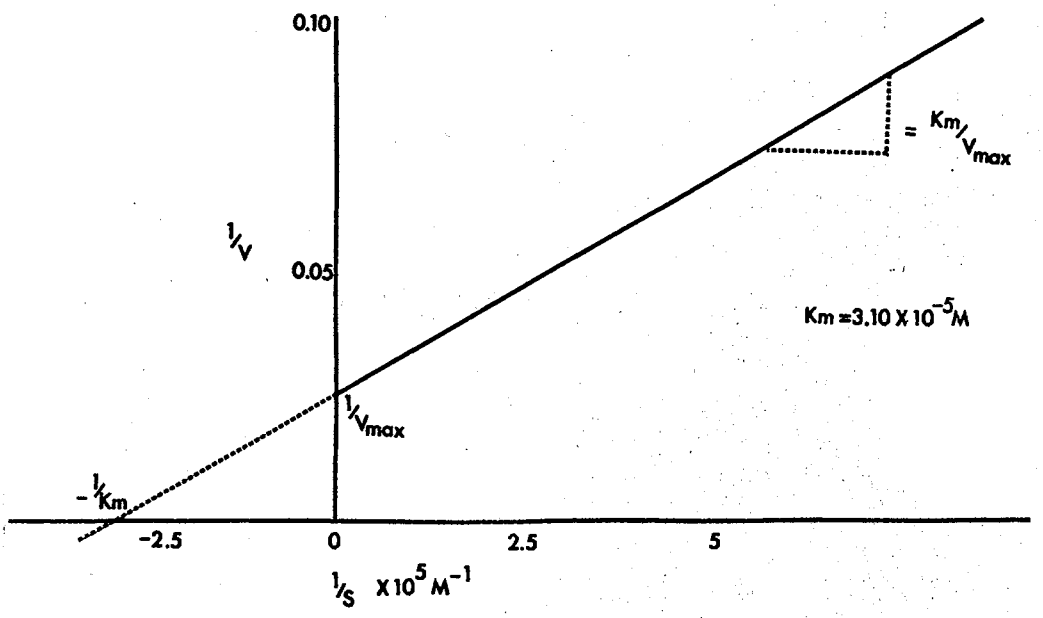


Figure 5. Lineweaver-Burk reciprocal plots of substrate versus lipase activity with induced 4-MUB fluorescence

over a limited pH range. In most cases a definite optimum pH is observed specific for that enzyme reaction. In this study it was found that the optimal pH for the wheat germ lipase 4-MUB reaction was 7.5 as shown in Table III and presented graphically in Figure 6. Troller and Bozeman (29) in their studies with lipase characterization of a Staphylococcus found that optimal pH is 7.5, while Fink and Koehler (6) have suggested a pH range of 6 to 8. Guilbault and Sader (8) proposed an optimum pH of 7.0 for this reaction.

The effects of temperature on most enzyme systems are usually complex. Both extremes of temperature retard the enzyme reaction. Table IV and Figure 7 show that an apparent optimum temperature for this reaction was 30°C. This confirmed results obtained by Troller and Bozeman (29) who determined the effects of temperature on purified lipase at 15 to 45°C. They found that optimum temperature was about 30 to 32°C for lipase reactions.

Although a number of cations or anions and their combinations were studied during this experiment, a few were found to have either an activating or an inhibiting effect. Ca^{++} as acetate salt at 1×10^{-1}M and Mg^{++} as acetate salt at 1×10^{-3}M had significant effects on activating the enzyme reaction, Table V. Zn^{++}, Pb^{++}, Hg^{+}, Ag^{+}, Al^{+++}, Fe^{++}, Co^{++}, and Mn^{++} as acetate salts had no significant effect on the wheat germ lipase 4-MUB reaction. Combinations of cations were highly significant, particularly Ca + Mg + K as shown in Table V. Na^{+} or K^{+} did not apparently have an effect on the reaction. Troller and Bozeman (29) also noted that the pure lipase activity increased by the addition of 10^{-3} and 10^{-4}M of Ca^{++}. They further reported that the other divalent cation Mg^{++}, produced similar results. Whereas

TABLE III

EFFECTS OF SOLUTION pH ON 4-MUB FLUORESCENCE INDUCED WITH WHEAT GERM LIPASE

pH levels	Fluorescence I	II	III	Ave.
6.5	25	26	25	25
7.0	30	28	30	29
7.5	37	37	37	37
8.0	30	32	32	31

Lipase level 3.3×10^{-3} units/ml, 4-MUB level 1.0×10^{-2}M

Treatment F: 77.74 ($P<.01$), EMS .91

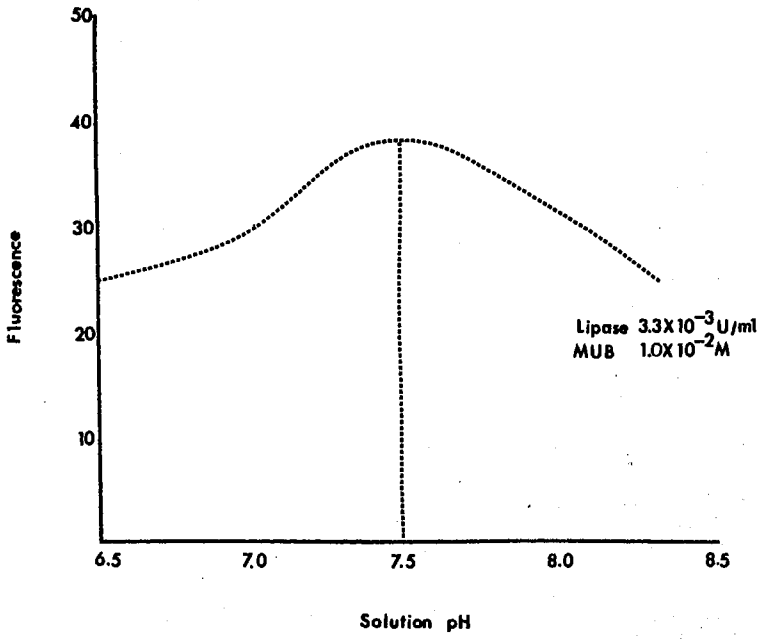


Figure 6. Effects of differential pH in solution on lipase activity with 4-MUB using 0.1 M phosphate buffer.

TABLE IV

EFFECT OF DIFFERENTIAL TEMPERATURE ON WHEAT GERM LIPASE ACTIVITY INDUCING MUB FLUORESCENCE

Temp. °C	Fluorescence I	II	III	Ave.
5	2	2	0	1.11
30	37	35	35	35.70
80	12	10	12	11.40

Treatment F = 842.73, (P<.01), EMS 1.11

Lipase level in all culture 3.3×10^{-3} units/ml, 4-MUB level 1.0×10^{-2}M

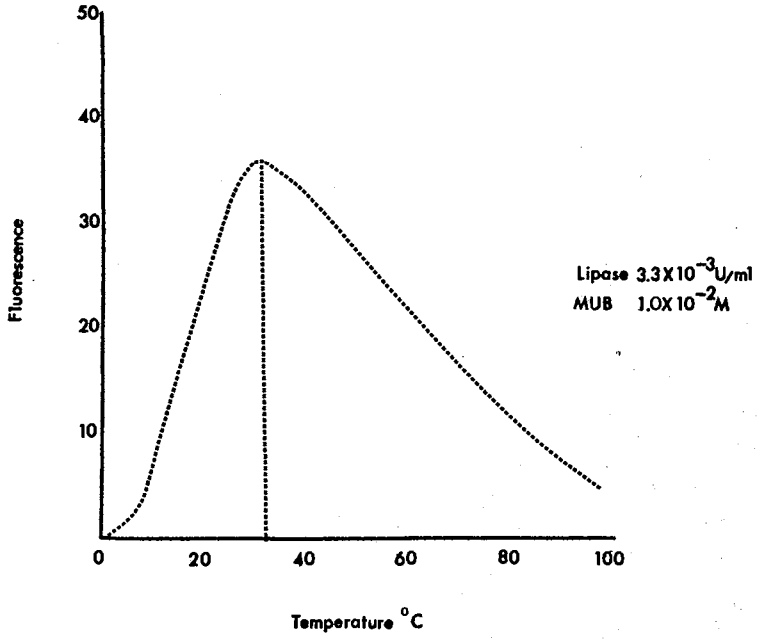


Figure 7. Effects of increasing temperature on wheat germ lipase activity with 4-MUB.

TABLE V

EFFECTS OF VARIOUS CATION CONCENTRATIONS AND COMBINATIONS ON ACCELERATION OF WHEAT GERM LIPASE INDUCED FLUORESCENCE OF 4-MUB

Cation-Level Moles per liter	Fluorescence I	II	III	Ave.
Ca^{++}				
1×10^{-1}	50	52	52	51
1×10^{-2}	32	34	34	33
1×10^{-3}	32	35	33	34
1×10^{-4}	37	34	33	35
1×10^{-5}	37	33	33	34
Mg^{++}				
1×10^{-1}	20	22	21	21
1×10^{-2}	23	23	24	23
1×10^{-3}	66	64	64	65
1×10^{-4}	22	22	22	22
1×10^{-5}	25	24	24	24
Cation combinations*				
Ca + Mg	50	51	51	51
Ca + Mg + Na	110	112	114	111
Ca + Mg + K	114	114	114	114
Ca + Mg + Na + K	106	106	105	106

*Ca 10^{-1}M, Mg 10^{-3}M, Na 10^{-1}M, K 10^{-1}M.

Lipase level in all cultures 3.3×10^{-3} units/ml with all cations as acetate salt. 4-MUB levels 1.0×10^{-2}M.
Treatment F: 2057.16, ($P<.01$), EMS 1.71

addition of NaCl to the reaction mixture produced a measurable decline in the hydrolysis of the tributyrin.

Anions gave variable results on the enzyme reaction. Cyanide and sulfide were found to be inhibitory in nature, whereas nitrate, nitrite, carbonate, bicarbonate, hydroxide, sulfate, and chloride did not have a significant effect on the enzyme reaction.

Five inhibitors in these studies, aflatoxin, cupric acetate, ferric chloride, sodium cyanide, sodium sulfide and ethylene diamine tetra acetic acid gave significant percent fluorescence inhibition as shown in Table VI. Aflatoxin inhibited reaction almost 86.9 percent whereas sodium sulfide inhibited only 24.6 percent. Troller and Bozeman (29) have reported that the sulfhydryl group inhibitor PCBM does not produce inhibition of tributyrinase activity at concentrations at which it might be expected to be an inhibitor at the active site. Black and Altschul (2) observed that the development of gibberallic acid-induced lipase activity could be completely inhibited with actinomycin D (50 µg/ml) in germinating cotton seedlings. Similarly aflatoxin at 45 µg/ml also inhibited the activity. According to these authors it was suggested that lipase activity in the germinating cotton seed is related to DNA-dependent RNA synthesis. Singer (23) reported the inhibition effect by sulfhydryl groups on lipase activity. EDTA and Cu^{++} have been shown to inhibit the reaction of hog pancreatic lipase. Cyanide as such is a biological poison and is expected to inhibit the enzyme reaction with Fe^{+++} acting similar to EDTA in chelation reactions.

Fluorescence visualization with long wave UV light is shown in Figure 13 with polaroid recordings of wheat germ lipase-MUB reaction.

TABLE VI

EFFECTS OF VARIOUS INHIBITORS ON WHEAT GERM LIPASE INDUCED FLUORESCENCE OF 4-MUB

Inhibitors at 10^{-1}M	% Fluorescence Inhibition I	II	III	Ave.
Aflatoxin (120 µg)	86.9	86.9	86.9	86.9
Cu (ic) acetate	56.5	60.8	60.8	59.4
Fe (ic) chloride	78.3	78.3	78.3	78.3
Na_2CN	56.5	52.2	56.5	55.1
Na_2S	21.7	26.0	26.0	24.6
EDTA	56.5	56.5	56.5	56.5

Treatment F: 457.82, ($P<.01$), EMS 3.08

Lipase level in all cultures 3.3×10^{-3} units/ml with percent inhibition determined as: $\frac{\text{check (no inhibitor) - inhibitor}}{\text{check (no inhibitor)}} \times 100$

Highly fluorescent spots were recorded with 5 X 10^{-3} and 2.5 X 10^{-3} units of wheat germ lipase. Fluorescence was directly proportional to the concentration of wheat germ lipase applied. Chang and Lynd (4) used this same technique to record aflatoxin fluorescence with thin layer chromatography separations.

Initial studies to characterize soil lipase were with Pokorna's (20) method and yielded the results shown in Table VII. A linear relationship f(x) 0.92 was established between the 3 percent olive oil added soil with 15 days incubation and the milliequivalents of fatty acids produced. This method of titrating the fatty acids has been widely used (20, 24, 29), but requires large amounts of soil extract and differences as a function of treatment are small and eratic. The presence of other lipids or salts alter the end point color. The results shown in Table VIII confirmed that although various treatments were given to this soil, no appreciable differences as a function of treatment could be obtained by the fatty acid titration method. Hence fluoremetric methods were used to determine lipase activity as suggested by Guilbault and Sadăr (8) and Jacks and Kircher (10).

Guilbault, et al. (7) evaluated various fluorometric substrates for lipase and suggested 4-methyl umbelliferone butyrate as one of the better substrates for lipase assay. Induced 4-MUB fluorescence with varying amounts of soil extract were determined and a significant difference was found in those observations, Table IX. Fluorescence was a linear function on soil lipase levels with f(x) 3.05 and r 0.97. Enzyme activity was destroyed with 100 µl of 10 percent TCA per culture or heating soil extracts to 80°C for 5 minutes. Trichloroacetic acid has been widely used to precipitate the proteins, of course in this

TABLE VII

LIPASE ACTIVITY FROM INCREASED SOIL ALCOHOL-ETHER EXTRACTS DETERMINED WITH OLIVE OIL HYDROLYSIS, EUFAULA SAND

Soil Grams	Meq. of fatty acids produced I	II	III	Ave.
1	0.3	0.3	0.3	0.30
2	0.6	0.6	0.6	0.60
3	1.0	0.9	0.9	0.93
4	1.2	1.2	1.2	1.20
5	1.5	1.6	1.5	1.53
6	1.8	1.8	1.9	1.83
7	2.0	2.1	2.1	2.07
8	2.5	2.5	2.5	2.73
9	2.9	2.8	2.7	2.80
10	3.1	3.0	3.0	3.03

Treatment F: 1301.5 ($P<.01$), EMS .002, $f(x) = .92$, $r = 0.99$
The soil was amended with 3% olive oil and incubated 15 days at 30°C in saturated humidity before lipase extraction.
1.5 meq fatty acid produced per 1 unit wheat germ lipase (Singer) in this procedure.

TABLE VIII

EFFECTS OF VARIOUS TREATMENTS ON SOIL LIPASE ACTIVITY AT THREE pH LEVELS, EUFAULA SAND

Soil Treatment	Soil Reaction pH 6.00	pH 6.60	pH 7.50	Ave.
	meq of fatty acids produced/g soil			
Check	2.02	1.96	0.79	1.59
Fertilizer	2.99	2.79	0.74	2.17
2% peanut residue	2.21	2.70	0.62	1.84
2% residue + fertilizer	2.13	2.91	0.73	1.92
Rye	2.61	2.09	1.30	2.00
Rye + fertilizer	1.71	2.59	1.32	1.87
Ave.	2.27	2.50	0.91	

Fertilizer treatment: 200 ppm N (NH_4NO_3) and 100 ppm P ($Ca(H_2PO_4)_2 \cdot H_2O$)

Rye: 20 plants/pot for 15 days and then incorporated in soil

Lipase was determined by free fatty titrations with the olive oil hydrolysis method. H^+ contributed from other soil components, particularly in the acid soils, should contribute to errors in this procedure. Soil incubation was 30 days at 30°C.

Treatment F: 37.20, Soil Treatment 2.16, soil pH 29.93

Interaction 5,10, ($P = <.01$), EMS 0.169

TABLE IX

EFFECTS OF SOIL EXTRACTABLE LIPASE - PHOSPHATE BUFFER RATIOS ON INDUCED MUB FLUORESENCE

Soil Extract gram eqv.	Fluorescence I	II	III	Ave.
0.25	2	2	1	1.7
0.50	3	3	4	3.7
1.00	5	5	5	5.0
2.00	8	8	7	7.7
3.00	9	9	10	9.7

Lipase activity within other cultures duplicating the above treatments was destroyed by heating soil extracts to 80°C for 5 minutes or by addition of .1 ml 10% TCA. All soil cultures received 2% olive oil addition with 15 days incubation at 30°C before extraction.

Treatment F: 764.19 ($P<.01$), EMS .078, f(x) 3.05, r 0.97

case no precipitate was apparent but the enzymatic activity was completely destroyed. Troller and Bozeman (29) observed the decrease in lipase activity with increase in temperature.

Few investigations as such have been made to enrich specific soils and determine the subsequent lipase activity. McLaren and Estermann (17) revealed that the adsorption on the clay mineral reduces soil enzyme activity and portions of the additive becomes inert. However, this is generally true for the proteins which carry specific charges and are adsorbed on the clay micelle. During these studies, 0.1M phosphate buffer of pH 7.5 was used and hence it was assumed that all possible extracellular enzyme is deadsorbed from the clay and is in an active form in the soil extract. In these studies the enrichment of the soil was accomplished with high lipid, fat-like materials that are nonpolar and are independent in nature from the clay micelle charges.

Results in Table X show that soil lipase activity was increased as the amount of olive oil amendment was increased. Maximum activity was observed when 5 percent olive oil was added to the soil. Protein determinations of same fraction showed that as the amount of protein increased the enzyme activity also increased except at 5 percent where protein level remained almost constant but enzyme activity increased, maximum specific activity obtained for soil lipase was 1.6×10^{-2} units per mg of protein (Figure 8). This suggests a maximum level of 4 percent olive oil addition to soil. Between 1 and 2 percent olive oil addition the enzyme tripled and protein content was doubled. Hence these olive oil levels were considered to yield a very significant change and used further in these studies. In similar studies with uricalytic enzymes in soil Smith (25) enriched the soil cultures with

TABLE X

SOIL LIPASE AND PROTEIN LEVELS OF SOIL CULTURES WITH DIFFERENTIAL OLIVE OIL AMENDMENT AFTER 15 DAYS INCUBATION AT 30°C

% Olive Oil Added	Per 10 grams soil: Lipase units X 10^{-3} I	II	III	Ave.	mg protein I	II	III	Ave.
1	0.1	0.1	0.1	0.10	.04	.02	.03	0.030
2	0.3	0.2	0.3	0.27	.06	.05	.06	0.057
3	0.8	0.8	0.9	0.83	.06	.06	.07	0.063
4	1.1	1.0	1.0	1.00	.08	.07	.08	0.077
5	1.5	1.3	1.5	1.43	.08	.07	.07	0.073

Lipase determinations with induced MUB fluoresence and protein values are the increased values over the check nontreated soil.

Lipase treatment F: 14.00 ($P<.01$), EMS 0.04, f(t) 3.43, C.V. 9.32%
Protein f(t) 1.067, C.V. 11.39%

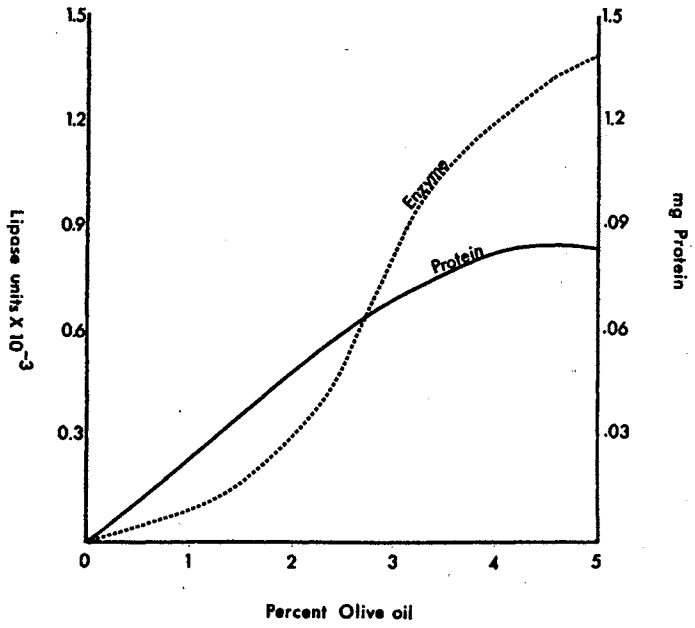


Figure 8. Relationship between soil lipase activity and extractable protein levels with increasing olive oil amendment to Eufaula sand.

sodium urate solution at 1mg uric acid/ml.

Soil organic matter (humus) has always been considered a potential source of nutrients as the substrate which soil microorganisms could utilize (28). However, McLaren and Estermann (17) have shown concern about the adsorption of such particles on clay mineral.

Addition of 1 and 2 percent olive oil to the soil with varying incubation periods yielded highly significant responses as shown in Table XI. The maximum lipase activity in case of 1 percent olive oil addition was reached after 20 days of incubation and then declined (Figure 9). The quadratic function was f(x) 0.357 - .008x. However, the 2 percent olive oil amended soil showed continued increases in lipase activity to 30 days with a linear function of f(x) 0.239.

Peanut residue addition at 1, 2, and 4 percent to the soil also yielded highly significant increases in lipase activity. The first five days indicated low activity of the lipase at the 2 and 4 percent levels. However, these levels of peanut residue, which contained 6.28 percent fat, induced high lipase activity after 10 days and the 4 percent level almost doubled the lipase activity between 25 to 30 days period of incubation, Figure 10. The 1 percent level was quadratic with f(x) 0.152 - .004x. The 2 percent level was linear with f(x) 0.054 and 4 percent linear with f(x) 0.111. These results indicated that olive oil was readily available to soil lipase producing microorganisms as a nutrient source whereas the peanut residue apparently required an initial period of decomposition of several days before an appreciable change in lipase activity was observed. This indicates biological activity rather than nonbiological chemical reactions between the additives and soil components. Similarly, Casida, et al.

TABLE XI

EFFECTS OF INCUBATION TIME AND HIGH LIPID AMENDMENT ON SOIL LIPASE LEVELS

	Soil Amendment				
	Olive Oil		Peanut Residue		
Days	1%	2%	1%	2%	4%
	Lipase units X 10^{-4} per g soil				
5	0.58	0.81	0.61	.01	.01
10	0.93	1.76	1.53	1.89	2.11
15	1.97	2.33	1.37	1.44	2.67
20	3.15	3.98	1.37	2.06	3.07
25	2.70	5.98	1.17	1.89	3.71
30	1.86	6.25	0.87	3.19	5.93

Figures are average lipase increases of six cultures over their corresponding check soil cultures incubated at 30°C, determined by MUB fluorescence. F values: Treatment level 55.65 ($P<.01$), Time 54.41 ($P<.01$), interaction 9.46 ($P<.01$), EMS 0.95. Check soil lipase values increased from 0 to 0.76 $\pm$ 0.012 U X 10^{-4} at 30 days with f(x) .028 with linear F 14.56 ($P<.01$). Olive oil 1%, quadratic F 52.30 ($P<.01$) f(x) .357 - .008x and 2%, linear F 5.53 ($P<.05$), f(x) .239. Peanut residue 1%, quadratic F 54.19 ($P<.01$), f(x) .152 - .004x; 2%, linear F 164.98 ($P<.01$), f(x) .054 and 4%, linear F 95.20 ($P<.01$) f(x) .111x.

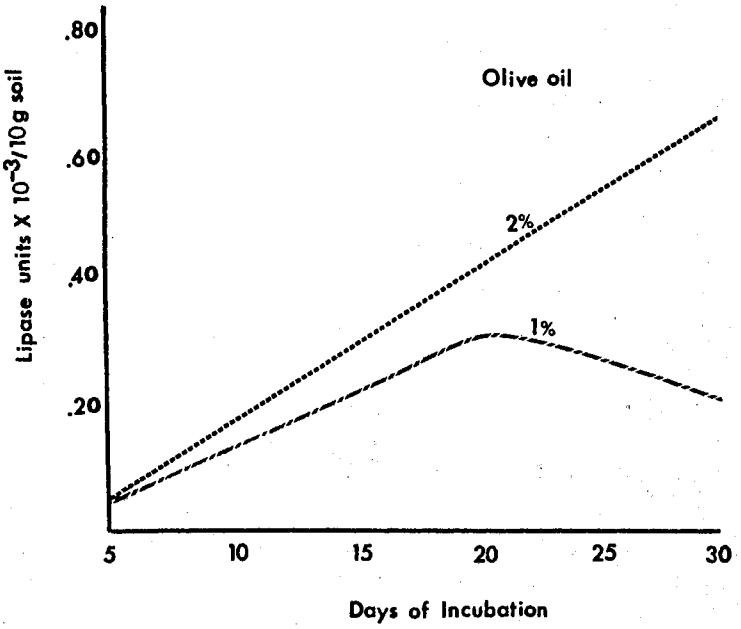


Figure 9. Effects of time on increased soil lipase activity with two olive oil amendment levels to Eufaula sand.

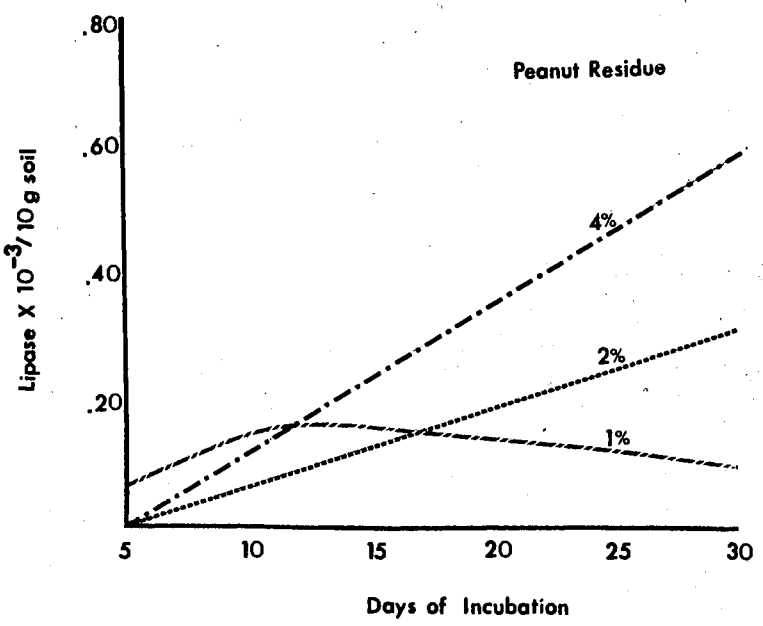


Figure 10. Effects of time on increased soil lipase activity with three peanut residue amendment levels to Eufaula sand.

(3) using 2, 3, 5 - triphenyltetrazolium chloride measuring soil dehydrogenase activity showed that tetrazolium reduction was biological in nature and was not a biologically independent chemical reaction.

Although olive oil was apparently effective for inducing increased soil lipase activity, there is much interest as to the relative effectiveness of other natural plant oil concentrates amended to soil in this same manner.

Results with five oils, having contrasting fatty acid compositions, amended at two levels at three soil pH levels are presented in Table XII. Olive oil additions resulted in the highest detectable soil lipase activity of the five oils used at all three pH levels and at both 1 and 2 percent additions. Olive oil contains about 88 percent unsaturated fatty acids of which some 80 percent occurs as C_{18-2H} (Oleic). Corn oil induced high lipase activity at all three soil pH and levels of soil amendment. Although the corn oil unsaturated acid composition also totals about 88 percent, 55 percent occurs as C_{18-4H} (Linoleic). Safflower oil was also among the effective lipase inducers with 92 percent unsaturated oils, 76 percent as C_{18-4H} (Linoleic). Peanut oil was apparently less effective than the three previously mentioned oils in these studies. About 20 percent of the peanut fatty acids were saturated mostly as C_{16} (Palmitic) and C_{18} (Stearic). Lowest activity resulted with coconut oil additions at all levels of amendment and soil pH. Over 90 percent of the fatty acids of coconut oil are fully saturated. Rapidity of microbiological degradation with an accelerated proliferation of lipase producers was apparently associated with the amount of unsaturation in fatty acid composition of the soil amendments.

The effects of soil pH on activity of lipase producers was significant as shown in Table XII. Lipase activity was lowest at the strongly acid soil pH 4.8 and highest at the alkaline soil pH 8.6. Lowest enzyme activity was attained with the coconut oil amendments in the acid soil and highest with olive oil additions in the alkaline soil.

Generally, base saturation of mineral soils with Ca^{++}, Mg^{++}, K^{+} and Na^{+}, along with the chemical activity of high iron levels are important factors in soil fertility. Addition of cations, Ca^{++}, Mg^{++}, K^{+} and Na^{+}, and their combinations yielded results similar to that obtained with wheat germ lipase. Among individual cations, the addition of Ca^{++} to the soil at a level of 4×10^{-4}M gave maximum soil lipase activity (Table XIII) followed by Ca^{++} at 2×10^{-4}M and Mg^{++} at 4×10^{-4}M. Studies with wheat germ lipase as described earlier (7) and with soil lipase (20) indicate that one of the most important cation which can increase soil lipase activity is Ca^{++}. Neither Na^{+} nor K^{+} gave any appreciable increase in lipase activity. Electrical conductivity was determined in order to check apparent soil salinity effects that may result with these cation levels but in all cases it was quite low. Cation level combinations of Ca + Mg or Ca + Mg + Na or Ca + Mg + K or Ca + Mg + Na + K were almost equally effective, Table XIII. This indicated that generally it was the divalent cations as Ca^{++} and Mg^{++} which were responsible for increasing lipase activity rather than monovalent cations as Na^{+} and K^{+}. Soil pH when determined of these treated soils was found to be near 7.0 to 7.1, hence any effect due to change in pH should be of minor importance. Ladd and Butler (15), while determining the effect of monovalent and divalent cations on proteases, showed that enzyme activity was greatly increased by

TABLE XII

EFFECTS OF OIL AMENDMENT WITH CONTRASTING FATTY ACID COMPOSITION ON SOIL LIPASE ACTIVITY

Treatment	Acid Soil (pH 4.8) 1%	Acid Soil (pH 4.8) 2%	Neutral Soil (pH 7.1) 1%	Neutral Soil (pH 7.1) 2%	Alkaline Soil (pH 8.6) 1%	Alkaline Soil (pH 8.6) 2%
	Lipase units X 10^{-3}/10g soil					
Peanut Oil	0.52	1.26	1.78	2.85	1.67	2.82
Coconut Oil	0.29	0.96	0.89	1.51	0.80	1.63
Safflower Oil	0.86	1.34	2.18	3.33	1.83	4.06
Corn Oil	0.88	1.38	1.76	2.86	1.86	4.17
Olive Oil	0.89	1.99	2.50	4.33	2.96	5.27

Figures are means of triplicate cultures as increased soil lipase activity over their corresponding check soil cultures at each pH level, Eufaula soil incubated 15 days at 30°C in saturated atmosphere.

Treatment F: 79.53 ($P<.01$), EMS 0.071

TABLE XIII

EFFECTS OF VARIOUS CATION LEVELS AND COMBINATIONS ON SOIL LIPASE ACTIVITY WITH OLIVE OIL AMENDMENT

	Cation level M X 10^{-3}		
	0.1	0.2	0.4
	Lipase units X 10^{-3} per 10 g soil		
Ca	1.09 (130)	3.06 (198)	3.13 (307)
Mg	0.72 (83)	0.93 (150)	1.26 (207)
Na	0.55 (68)	0.53 (108)	0.59 (180)
K	0.52 (147)	0.67 (160)	0.69 (213)

Cation level combinations at 0.2 M X 10^{-3}

	0	Na_2	K_2	Na_2K_2
Ca_2+Mg_2	3.39 (197)	3.66 (286)	3.59 (517)	3.58 (583)
Ca_2	3.06 (198)	2.83 (203)	2.77 (183)	
Mg_2	0.93 (150)	1.96 (218)	1.98 (353)	

Figures are means of triplicate cultures with 15 days incubation of 30°C determined by MUB fluorescence differences between olive oil amended soil cultures and nonamended soil culture with the same corresponding cation levels. Treatment F value: 396.588 ($p<.01$), EMS 1.187.

All salts were added as chlorides. Soil pH was near neutral 7.0-7.1 Electrical conductivity as mmhos $10^{-5}/cm^2$ of soil solution are presented in parenthesis.

addition of a divalent cation as Ca^{++} and Mg^{++} at 1 X 10^{-2}M. Whereas monovalent cation, including Na^{+} and K^{+} as chlorides at 1 X 10^{-2}M, did not produce the same increase in activity as by divalents, although KCl was more effective than Na.

Effect of inhibition of enzyme reaction was determined as percent fluorescence inhibition as described in Chapter III. In general, inhibition was increased as the substrate concentration was decreased as shown in Table XIV. This was particularly true when inhibitor concentration was 1 X 10^{-1}M as shown in Figure 11. EDTA, Cu^{++}, S^{--} blocked the reaction completely at an inhibitor level of 1 X 10^{-1}M and substrate level 1.25 X 10^{-3}M. The above mentioned three inhibitors could be considered partially competitive in nature for the substrate combining with the active enzyme sites because addition of substrate reduced this inhibition. However, Fe^{+++} was apparently independent of substrate concentration (Figure 11) and resulted in almost a constant inhibition of 50% of the reaction, this suggests Fe^{+++} to be noncompetitive in nature.

When low concentration of inhibitor, 1 X 10^{-5}M was used, reverse results were obtained except in case of S^{--}, where 100% inhibition of reaction was observed with lowest level of substrate, 1.25 X 10^{-3}M, Figure 12. In case of EDTA and Fe^{+++} the percent inhibition decreased to a minimum with decreasing concentration of substrate. In case of Cu^{++}, inhibition was only 20%, whereas S^{--} still had 100% inhibition. This possibly indicates a dilution phenomenon with Cu^{++}. Figure 12 and Table XIV indicated that S^{--} was highly competitive in nature and inhibition was still 100% even after the concentration of S^{--} was reduced to 1 X 10^{-5}M.

TABLE XIV

EFFECT OF SUBSTRATE AND INHIBITOR CONCENTRATIONS ON SOIL LIPASE INDUCED 4-MUB FLUORESCENCE

Inhibitor conc.	Substrate conc.	EDTA	Fe^{+++}	Cu^{++}	S^{--}
		% Fluorescence Inhibition			
$1 \times 10^{-1}M$	$1 \times 10^{-2}M$	20.4	56.7	30.3	20.2
	$5 \times 10^{-3}M$	30.3	49.5	9.7	14.0
	$2.5 \times 10^{-3}M$	50.3	47.4	21.0	30.3
	$1.25 \times 10^{-3}M$	100.0	48.3	100.0	100.0
$1 \times 10^{-5}M$	$1 \times 10^{-2}M$	17.5	22.4	2.7	1.06
	$5 \times 10^{-3}M$	15.0	14.0	2.1	17.1
	$2.5 \times 10^{-3}M$	0	14.0	26.3	17.1
	$1.25 \times 10^{-3}M$	0	8.3	20.3	100.0

Treat F ratio: 351.83 ($P<.01$), EMS 11.91. Salts used were ferric chloride, copper sulfate and sodium sulfide. Figures are means from three replicate cultures with % inhibition = Fluoresence of

$$\frac{\text{check culture-treated culture}}{\text{check culture}} \times 100$$

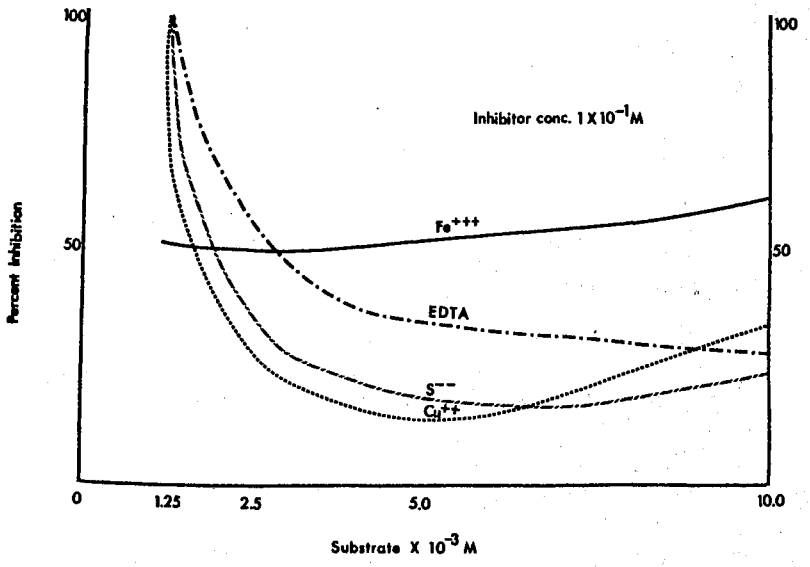


Figure 11. Effects of increasing substrate levels on 0.1 M inhibitor concentrations with 3.3 X 10^{-3}U lipase.

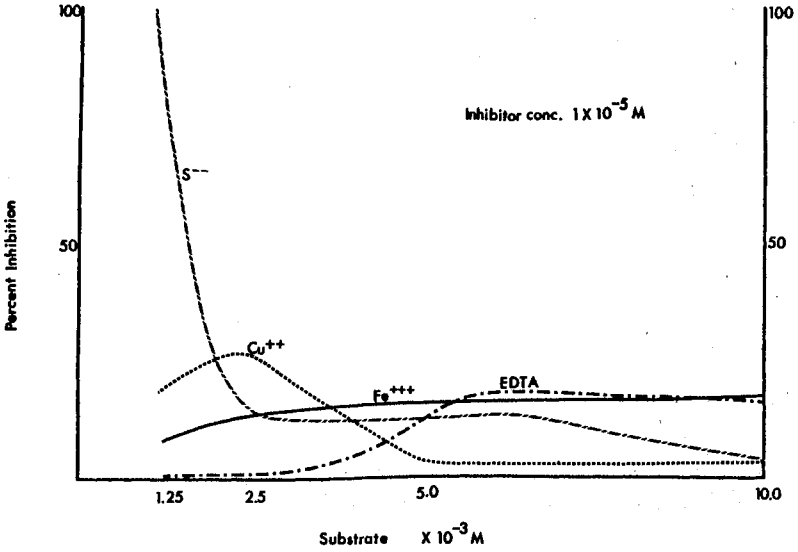


Figure 12. Effects of increasing substrate levels on 1 X 10^{-5}M inhibitor concentrations with 3.3 X 10^{-3}U lipase.

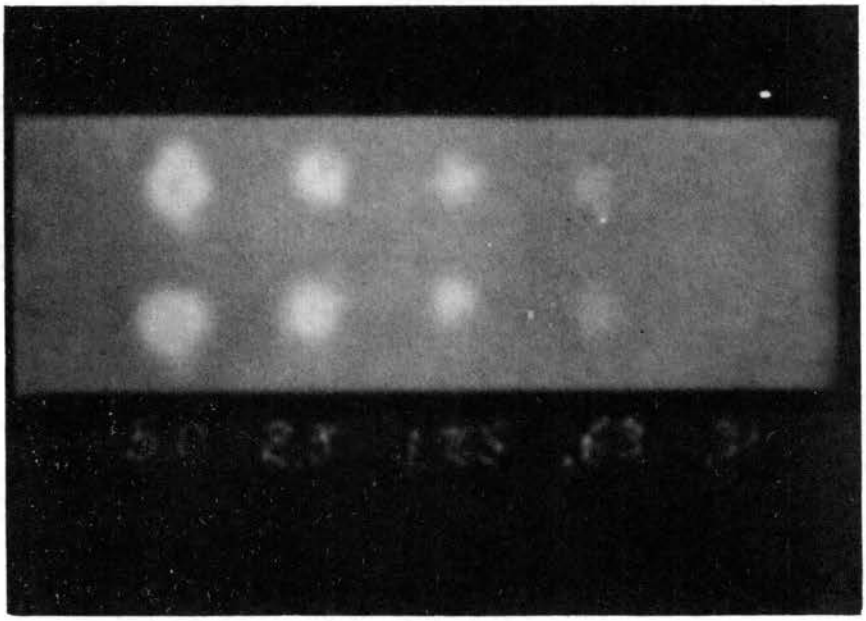


Figure 13. Polaroid recording of UV fluorescence induced with 5 µl spots of .31, .63, 1.25, 2.5 and 5.0 x 10^{-3}U wheat germ lipase (Singer) with 4-MUB saturated glass fiber.

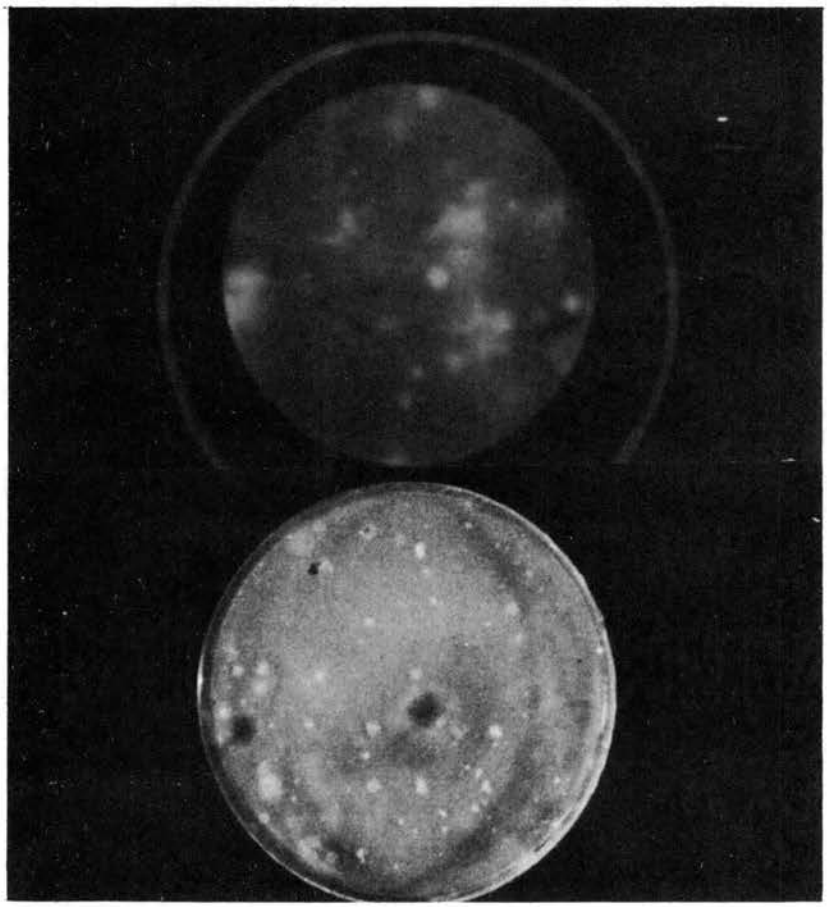


Figure 14. Culture plate that received lipid enriched soil extract dilution equivalent to 6.0 µg soil. Developed soil microbial colonies (lower), colony extracellular lipase indicated with MUB fluorescence (upper).

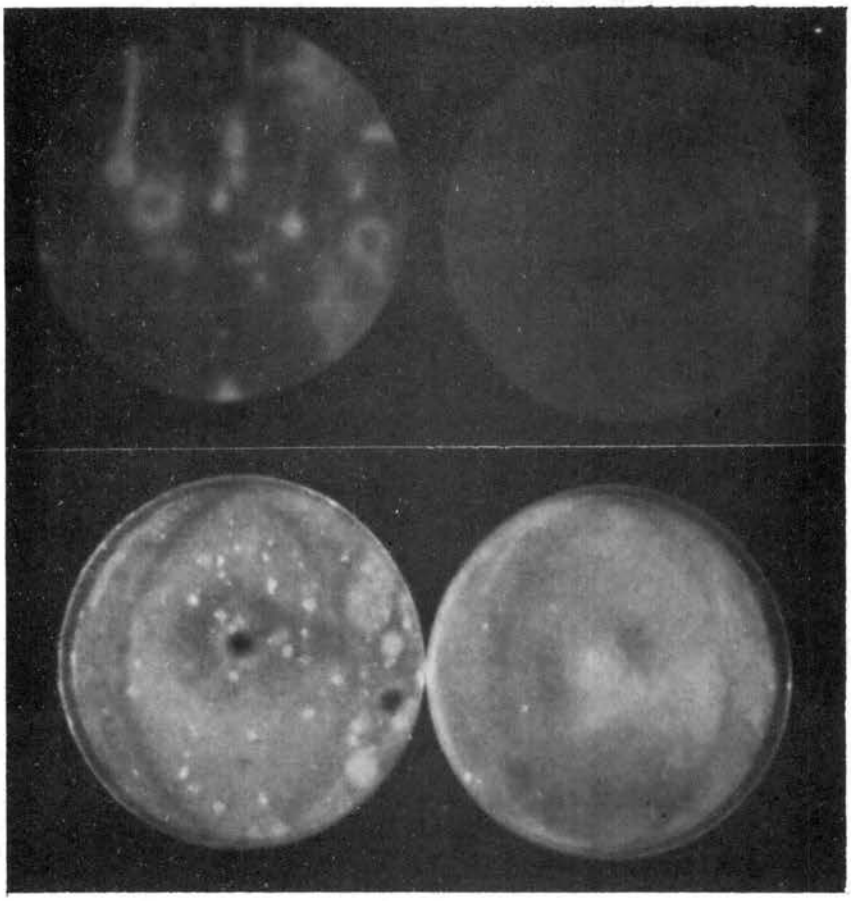


Figure 15. Culture plates receiving nonenriched and enriched soil extract dilutions equivalent to 6 µg soil are shown with corresponding MUB fluorescence developing using saturated glass fiber filters.

Visual examination of various plates for growing the soil microflora indicated significant differences among nontreated soil and enriched soil microflora. In nontreated soil, mostly fungi dominated with few actinomycetes and bacterial colonies appearing in those plates.

Plates which received diluted soil solution from enriched soil (4% olive oil) had few fungi and generally a large number of bacterial colonies with actinomycetes also present in appreciable numbers. Some of the treated as well as nontreated plates were screened for lipase activity with 4-MUB saturated glass fiber filter as described in Chapter III. Figure 14 shows the comparison of one of these plates before and after addition of 4-MUB saturated glass fiber filter. The plate which received enriched soil dilution exhibits distinctive microbial colonies and the corresponding MUB treated plate shows the fluorescence induced from extracellular lipase of these colonies. This indicated confirmation of the reaction of extracellular lipase from cultures which split 4-MUB to highly fluorescent 4-MU. Figure 15 shows the culture plates side by side before and after saturating them with 4-MUB. These plates which received soil dilutions equivalent to 6.0 µg of soil, showed distinctive colonies of bacteria, actinomycetes and fungi when enriched soil solution was used. Whereas in nontreated soil very little microbial growth was observed at this dilution. Plates with colonies having extracellular lipase again produced high fluorescence as in case of Figure 14.

CHAPTER V

SUMMARY AND CONCLUSION

The objective of this study was to determine parameters of soil lipase activity using 4-Methyl Umbelliferone Butyrate. Characterization of wheat germ lipase (Singer) indicated Km = 3.1 X 10^{-5}M, Vmax 36.3 fluorescence units/3 minutes, and optimum temperature of 30°C with pH 7.5. Ca^{++} and Mg^{++} were found to accelerate lipase activity at 1 X 10^{-1}M to 1 X 10^{-5}M concentration. Aflatoxin at 120 µg per culture, Cu^{++}, EDTA, Fe^{+++}, CN^{-} and S^{--} all at 1 X 10^{-1}M were apparent inhibitors of the wheat germ lipase- MUB reaction. The fluorometric method of lipase determination was approximately one thousand fold more sensitive than the standard fatty acid titration method and thus could detect very low levels of lipase activity present in soil.

Soil lipase activity was highest at an optimum pH 7.5 and temperature of 30°C with maximum specific activity 1.6 X 10^{-2} lipase units per mg protein. Lipase activity was increased to high levels by soil addition of highly unsaturated plant oils at levels of 1 or 2 percent. A delayed but enhanced activity of soil lipase was observed when peanut residue was added to the soil at 1, 2 and 4 percent. Maximum soil lipase activity was observed with these soil amendments after twenty days of incubation at 30°C. Ca^{++} and Mg^{++}, both at 4 X 10^{-4}M, were accelerators of the liplytic reaction. Na^{+} and K^{+} did not have any effect on the reaction, whereas combination of two divalent cations

i.e. Ca^{++} + Mg^{++} were additive and almost doubled the lipase activity. Cu^{++}, EDTA, Fe^{+++} and S^{--} inhibited the reaction at 1 X 10^{-1}M. All of the inhibitors except Fe^{+++} were apparently competitive in nature.

Examination of induced 4-MUB fluorescence on agar plates which received diluted soil extracts indicated that lipase producers were principally bacteria and actinomycetes. Extracellular lipase activity was confirmed with induced MUB fluorescence using polaroid recordings.

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VITA

Sunil Kumar Pancholy

Candidate for the Degree of

Doctor of Philosophy

Thesis: QUANTITATION OF SOIL LIPASE WITH 4-MUB FLUORESCENCE

Major Field: Soil Science

Biographical:

Personal Data: Born in Udaipur, Rajasthan, India, July 28, 1945, son of Sobhagya N. and Nalini Pancholy.

Education: Graduated from Raj. Coll. Agri., Udaipur, Rajasthan, India, in July, 1961; received the Bachelor of Science degree from University of Udaipur with a major in Agriculture in July, 1964; received the Master of Science degree from University of Udaipur, with a major in Agronomy in July, 1966; received the Master of Science degree from Oklahoma State University, with a major in Agronomy in July, 1968; graduate study toward doctoral degree at Oklahoma State University with a major in Soil Science, September, 1968 to May, 1971.

Experience: Recipient of Junior Fellowship of Council of Scientific and Industrial Research, New Delhi, India, 1966. Research Assistant at Indian Agriculture Research Institute, New Delhi, July, 1966 to January, 1967; part-time lab technician, February, 1967 to August, 1967 at Oklahoma State University; Graduate Research Assistant, Oklahoma State University, September, 1967 to January, 1971.

Member of: Indian Society of Agronomy, Indian Society of Soil Science, Young Farmers Association, American Society of Agronomy, Soil Science Society of America, and Sigma Xi.