Arbige-Neubeck 1-4

INSTRUCTIONS

(a) If Convention application insert "Convention"

(a) Convention

COMMONWEALTH OF AUSTRALIA

Patents Act

APPLICATION FOR A PATENT

(b) Insert FULL name(s) of applicant(s)

I/We (b) GENENCOR INC.

595481

(c) Insert FULL address(es) of applicant(s)

of (c) 180 Kimball Way
S. SAN FRANCISCO, CALIFORNIA, UNITED STATES OF AMERICA

hereby apply for the grant of a Patent for an invention entitled

(d) Insert TITLE of invention

(d) A LIPOLYTIC ENZYME DERIVED FROM A ASPERGILLUS MICROORGANISM HAVING AN ACCELERATING EFFECT ON CHEESE FLAVOR DEVELOPMENT

which is described in the accompanying (e) complete specification.

*(**Note:** The following paragraph applies **only** to Convention applications)*

This application is a Convention application and is based on the basic application(s) for a patent or similar protection identified by number, country, and filing date as follows:

(f)

623,931 UNITED STATES OF AMERICA 25 June 1984

FEE STAMP TO VALUE OF $ 205 ATTACHED
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19 JUN 1985
Melbourne

Address for Service: PHILLIPS, ORMONDE AND FITZPATRICK
Patent and Trade Mark Attorneys
367 Collins Street
Melbourne, Australia

APPLICATION ACCEPTED AND AMENDMENTS ALLOWED 12-1-90

(g) Insert DATE of signing

Dated (g) May 20, 1985

(h) Signature of applicant(s) (For body corporate see headnote*)

(h) Raymond C. Marier

(i) Corporate seal if any

(i)

Raymond C. Marier
Secretary

Note: No legalization or other witness required

AUSTRALIA

Patents Act

DECLARATION FOR A PATENT APPLICATION

▼ INSTRUCTIONS
(a) Insert "Convention" if applicable
(b) Insert FULL name(s) of applicant(s)

In support of the (a) CONVENTION application made by (b)

GENENCOR INC.

(c) Insert "of addition" if applicable
(d) Insert TITLE of invention

(hereinafter called "applicant(s) for a patent (c) for an invention entitled (d)

A LIPOLYTIC ENZYME DERIVED FROM A ASPERGILLUS MICROORGANISM HAVING AN ACCELERATING EFFECT ON CHEESE FLAVOR DEVELOPMENT

(e) Insert FULL name(s) AND address(es) of declarant(s) (See headnote*)

I/We (e) Mr. Paul W. Jones, Patent Attorney of Phillips Ormonde & Fitzpatrick, 367 Collins Street, Melbourne, Victoria, 3000, Australia.

do solemnly and sincerely declare as follows:

~~I am/We are the applicant(s)~~

(or, in the case of an application by a body corporate)

1. I am/We are authorized to make this declaration on behalf of the applicant(s).

~~I am/We are the actual inventor(s) of the invention~~

(or, where the applicant(s) is/are not the actual inventor(s))

(f) Insert FULL name(s) AND address(es) of actual inventor(s)

2. (f) MICHAEL VINCENT ARBIGE of 363 Thunder Circle, Bensalem, Pennsylvania, CLIFFORD EDWARD NEUBECK of 45 Horsham Road, Hatboro, Pennsylvania, both respectively of the United States of America

(g) Recite how applicant(s) derive(s) title from actual inventor(s) (See headnote**)

is/are the actual inventor(s) of the invention and the facts upon which the applicant(s) is/are entitled to make the application are as follows:

(g) Applicant is the assignee of the invention from the actual inventors

(Note: Paragraphs 3 and 4 apply only to Convention applications)

(h) Insert country, filing date, and basic applicant(s) for the/or EACH basic application

3. The basic application(s) for patent or similar protection on which the application is based is/are identified by **country, filing date, and basic applicant(s)** as follows:

(h) United States of America,
25 June 1984
MICHAEL VINCENT ARBIGE and CLIFFORD EDWARD NEUBECK

4. The basic application(s) referred to in paragraph 3 hereof was/~~were~~ the first application(s) made in a Convention country in respect of the invention the subject of the application.

(k) Insert PLACE of signing

(l) Insert DATE of signing

(m) Signature(s) of declarant(s)

Note: No legalization or other witness required

Declared at (k)

Dated (l)

(m) _____

Paul W. Jones

Patent Attorney

To: The Commissioner of Patents

P18/7/78

43835/85
KRH:MPS:GD

PHILLIPS ORMONDE & FITZPATRICK
Patent and Trade Mark Attorneys
367 Collins Street
Melbourne, Australia

(12) PATENT ABRIDGMENT (11) Document No. AU-B-43835/85
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 595481

(54) Title
LIPOLYTIC ENZYME FROM ASPERGILLUS FOR ACCELERATING CHEESE FLAVOR DEVELOPMENT

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(56) Prior Art Documents
US 3975544 A23C 19/12
US 4065580 A23D 5/00
CA 1091978 A23C 19/02

(57) Claim

1. A biologically pure culture of a variant of Aspergillus species known as ATCC No. 20719 as hereinbefore defined which produces a lipolytic enzyme having a tricaprylin to tributyrin hydrolysis ratio greater than 0.75.

3. A lipolytic enzyme derived from an organism belonging to the Aspergillus species known as ATCC No. 20719 as hereinbefore defined which has a tricaprylin to tributyrin hydrolysis ratio of at least 0.75.

4. An improved method of producing mild cheeses with characteristically low butryic acid content, the improvement comprising adding to the cheese curd or milk an effective

amount of lipolytic enzyme according to claim 3 having a tricaprylin to tributyrin hydrolysis ratio of at least 0.75, whereby the aging of the cheese is accelerated.

7. An improved method of producing cheese, the improvement comprising adding to the cheese curd or milk an effective amount of a first lipolytic enzyme according to claim 3 having a tricaprylin to tributyrin hydrolysis ratio of at least 0.75 and a second lipolytic enzyme with a tricaprylin to tributyrin hydrolysis ratio of less than 0.75.

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COMPLETE SPECIFICATION

(ORIGINAL)

Class Int. Class

Application Number:

Lodged:

Complete Specification Lodged:

Accepted:

Published:

Priority:

Related Art:

This document contains the amendments made under Section 49 and is correct for printing.

43835/85

APPLICANT'S REF.: Arbige-Neubeck 1-4 (A052001)

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Complete Specification for the invention entitled:

A LIPOLYTIC ENZYME DERIVED FROM A ASPERGILLUS MICROORGANISM HAVING AN ACCELERATING EFFECT ON CHEESE FLAVOR DEVELOPMENT

The following statement is a full description of this invention, including the best method of performing it known to applicant(s):

A LIPOLYTIC ENZYME DERIVED FROM A ASPERGILLUS MICROORGANISM HAVING AN ACCELERATING EFFECT ON CHEESE FLAVOR DEVELOPMENT

Technical Field

The present invention relates to a novel lipolytic enzyme derived from a novel Aspergillus microorganism. Cheese aged in the presence of a low concentration of this lipolytic enzyme ripens faster than with conventional lipolytic enzymes and without any lipolytic enzyme-associated rancidity.

Background Art

Accelerating of cheese aging to improve cost efficiency through a reduction of storage space is becoming more important to the cheese industry. Currently, lipases from two different sources, animal pregastric glands, and animal pancreases, are being added to cheese curd as accelerators. A third source, microbial lipases, has been used in commercial production of strongly flavored cheeses, but not mild cheeses.

Lipases derived from the pregastric gland of kid, calf and lamb are currently being added to accelerate ripening in cheese of Italian type (Provolone, Romano, Parmesan) where the characteristic rancid flavor of low molecular weight free fatty acids (e.g. butyric acid) is desirable. However, when these pregastric lipases

are used to accelerate mild flavored cheese, e.g. Cheddar, too much of the low molecular weight fatty acids are produced and a rancid flavor develops. When the animal pancreas lipases are used in a high concentration, excessive amounts of lauric acid is produced in the cheese, making a soapy taste. Moreover, unless highly purified, these lipases can contain an abundance of protease which although in small amounts aids cheese softening, in larger amounts it produces bitter off-flavors.

In the search for lipases showing desirable ripening characteristics, numerous organisms such as Pseudomonas fragii, P. fluorescens, Staphylococcus aureus, Clostridium lipolytica, Geotrichium candidum, Penicillium roqueforti, Aspergillus niger, A. flavus, A. oryzae, Candida cylindracea and Rhizopus oligosporous, have been used in laboratory studies. Many of these microbial lipases are not yet available commercially. In a study of various lipases for cheese ripening, Harper, W.J., [J. Dairy Science 40 556 (1957)], the following data has been developed with respect to selective liberation of free fatty acids from 20% butterfat cream.

Table 1

Release of Free Fatty Acid from 20% Butterfat Cream

Lipase Source	Mol. Butyric	% Produced in 3 hrs. @ 35 C Caproic	Caprylic	Capric	Lauric
Imported Kid rennet (paste)	32.8	11.3	7.1	11.8	33.6
Pregastric esterases					
kid	44.4	15.2	7.6	12.3	21.5
lamb	48.1	8.6	14.2	9.3	19.3
calf	36.7	8.9	14.8	10.7	39.0
Domestic calf rennet	10.7	3.1	trace	trace	86.5
Milk lipase	13.5	8.2	10.2	8.7	60.0
Pancreatic lipase	8.4	2.1	trace	trace	89.1
A. niger lipase	43.1	18.9	20.2	17.5	trace

Little information is available on the relationship between specific free fatty acids and flavor intensity in cheese although the presence of butyric acid usually is associated with strong rancidity development in strong cheese and the presence of large amounts of lauric may lead to a soapy flavor. Longer chain fatty acids have been linked to fruity flavors. It is generally noted that all of the even numbered free fatty acids are present but the concentration of the specific fatty acids varies in different types of cheese.

Disclosure of the Invention

The present invention relates to isolated cultures of a selected mutant strain of Aspergillus fungus. When cultured in a suitable nutrient medium, under proper aeration and agitation, it produces a novel lipolytic enzyme. For the purposes of this disclosure,

this type of enzyme shall be referred to as a lipase although esterases are included in this term.

Fungus

The instant fungus has been identified as a novel strain of Aspergillus. The present fungus has been deposited in the American Type Culture Collection and given the Accession Number 20719.

Colonies on Czapeks agar grow rapidly attaining a diameter of 5 to 6 cm in ten days at room temperature (24-26°C), with vegetative mycelium largely submerged but typically supporting a deep loose textured, surface growth of long-stalked conidial structures and intermixed aerial mycelium, at first white, then becoming yellow to olive-yellow.

Conidial heads radiate, mostly 150 to 300μ in diameter, rarely up to 400 or even 500μ, with conidial chains divergent on loosely adherent, conidiophores arising mostly from the submerged mycelium, colonies, typically long and up to 4 to 5 mm in length.

Colonies on malt extract agar grow even more rapidly and usually deeper than on Czapek's, sporulating somewhat more abundant and exhibiting shades near ecru-olive to rainette green, retaining the green pigmentation in age. Conidial structures loosely radiate as described above.

Method of Production of novel Aspergillus.

Organism from Wakesman collection in 1933
(natural isolate - characterised as
↓ A-oryzae
R+H stock culture (recharacterized as A-oryzae)
↓
NTG
mutagenisis round 1 1935
↓
NTG
mutagenisis round 2 1983
↘
screened for lipase production in cream substrates.

Enzyme

The present Aspergillus organism makes a novel and especially useful lipase when cultured in an oil-based medium the same manner as known Aspergillus organisms. However, the lipase from the present Aspergillus organism hydrolyzes longer chain C_6 and C_8 triglycerides such as tricaproin and tricaprylin more efficiently than short chain C_4 triglycerides such as tributyrin.

Lipase having this characteristic appears to be useful as a ripening accelerator in mild flavored cheese production, such as Cheddar cheeses. The less efficient hydrolysis of the shorter C_4 triglycerides suppresses the development of rancid flavors which are unwanted in mild cheeses.

When isolated from the present Aspergillus organism under proper dilution, agitation, pH, and salt concentration conditions, the present lipase is associated with low levels of protease which soften the cheese without producing bitter flavors.

Brief Description of the Drawings

FIGURE 1 is a graph showing the effect of mycelium concentration on lipase release.

FIGURE 2 is a graph showing the effect of temperature on lipase activity at pH 6.2.

FIGURE 3 is a graph showing the effect of pH on lipase activity at 33°C.

FIGURE 4 is a graph showing the effect of temperature on lipase activity at various pHs.

FIGURE 5a-f are graphs showing the effect of time on lipase activity at various temperatures and pHs.

FIGURE 6 is a graph showing the effect of time on tricaprylin hydrolysis by the present lipase at 40°C.

FIGURE 7 is a graph showing the effect of time on tricaprylin hydrolysis by the present lipase at 50°C.

FIGURE 8 is a graph showing the percent hydrolysis of a specific free fatty acid to total free fatty acid in enzyme-added cheeses.

FIGURE 9 is a graph showing the percent hydrolysis of a specific free fatty acid to total free fatty acid in enzyme-added cheeses.

Modes for Carrying Out the Invention

Culturing the Organism

The Aspergillus organism is stored on sterile soil under refrigeration. Portions of the soil stock are transferred to potato dextrose agar (PDA) and grown 4-5 days at 20-35°C, 30°C preferably, to use as primary inoculum for shake flask and fermenter growth.

Lipase can be made in a shaker flask by inoculating directly from the PDA slant into 100 ml. of sterile medium at pH 5.1 (5.0-5.2) containing 6% soy bean meal, 5% monobasic ammonium phosphate, 0.5% Magnesium sulfate (crystal), 2% soy oil, 1L Erlenmeyer flasks. After 3-5 days at 30°C and an agitation of 265 RPM, the culture is filtered. Preferred ranges for culture ingredients are: 1-10% oil; >.1-1% sulfate; 1-5% phosphate; and 3-8% soybean meal. The concentrations of $MgSO_4$, $(NH_4)H_2PO_4$ and oil is important to optimum production of lipase activity. The initial pH must not be below 5.0.

Production in a fermenter requires some modifications. The medium components are as described for shake flasks, but it is necessary to use an intermediate step of inoculum to reduce the growing time to the interval of 3-5 days. The inoculum volume used is 5% (1 to 10) of a nutrient containing 4% whole wheat flour and 8% corn steep water at pH 5.5. The sterile inoculum nutrient is inoculated with the PDA slant and grown 30-50 hrs. at 30°C and then transferred to the growing medium. Here, the oil may be corn or soy oil and the concentration may be from 1-6%. All of the oil can be present initially or added in increments after growth has started.

Preparing the Lipase

Filtering the fungal mycelium effectively isolates the lipase. The lipase activity of the crude culture when harvested (pH 4.5-5.0) is bound to the mycelium. The mycelium-free culture filtrate contains essentially no lipase activity (<5%), and all soluble extracellular components which may have a negative effect on cheese flavor.

Release of lipase activity from the mycelium into a second extraction filtrate depends on the concentration of the mycelium, the pH, the buffer salt concentration of extraction filtrate (0.5-3.0% PO_4 at optimum pH), the concentration of surfactent (preferably <1%), and the treatment time. The test results are obtained at a pH of 6.2, at 4°C where the mycelium is diluted 4%, adding 0.2% surfactant, and the extraction is carried out for 18-24 hours. This gives approximately 70% release from cells. No lysis of the cells occurs.

Fig. 1 illustrates how the amount of lipase released to extraction filtrate using Ethofot 242/25 surfactants varies according to the concentration of mycelium. All of this data was obtained at pH 6.2. Optimal recovery of lipase from mycelium is achieved normally at pH 6.2 because the lipase (free from mycelium) shows excellent stability for long periods. As the pH increases the lipase is less stable, and it is actively destroyed above pH 7.5. For example, after 42 hours at 4-5°C filtrate showed 94%, 87% and 74% activity retention respectively at pH 6.3, 6.8 and 7.4. After treatment under optimal conditions, about 80-90% of the lipase activity can be recovered from the mycelium filtrate.

The lipase in the extraction filtrate can be used directly for cheese flavor modification or alternatively concentrated in vacuo or by ultrafiltration before cheese addition. The filtrate or concentrate can be dried in vacuum at 0-35°C or spray dried to produce a dry concentrate which can be blended with other components, e.g. sodium chloride, phosphates, etc., used routinely in cheese manufacture. The methods of concentration and drying are conventional techniques known to those of ordinary skill in the art.

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Assaying Lipase Activity

Two different assays have been used for lipase activity. In the first, potentiometric titration is performed so as to determine lipase forestomach units (LFUs). One LFU equals the activity that releases 1.5 μ mol of butryric acid per minutes, Food Chemical Codex 3rd Ed., National Acadamic Press, 1981.

The titation substrate is prepared by dispering an amount of sodium caseinate equivalent to 600 mg of casein, in 95 ml of water contained in a one-half pint freezer jar that fits the head of a suitable high-speed blender. This is mixed with 0.5 gm of hydroxylated lecithin. Finally 5.0 ml of Tri-n-butyrin is added and mixed for 60 sec. at low speed. This substrate must be held to 33°C and used within 4 hours.

The sample is prepared by suspending or dissolving an accurately weighed amount of enzyme in water.

To measure, the titrator is filled with 0.05 N sodium hydroxide, and the instrument is calibrated following the manufacturer's instructions. The substrate is mixed for about 15 sec with a magnetic stirrer, then 20.0 ml is pipetted into the reaction vessel of the titrator. One ml of the sample is added and equilibrated for 15 min. The rate, in ml per min., at which the titrant was delivered during the titration, is determined and recorded as R. The activity of the enzyme is calculated by the formula:

$$LFU/g = R \times 0.025 \times 10^3/(W \times 1.25),$$

in which W is the weight, in g, of the enzyme preparation contained in the 1.0ml of Sample taken for analysis.

It should be noted that the enzyme's ability to hydrolyze tricaproin (C6), tricaprylin (C8), tricaprin (C10), can be measured simply by replacing tri-n-butyrin with the desired substrate.

A second assay is the cream-acid titration method. An aliquot (20.0 ml) of 10.5% weight/volume butterfat Half and Half is placed into 2 oz. glass jars and brought to 33°C in thermostated water bath. After temperature equilibrium has been established (about 10 minutes), 1.0 ml of enzyme solution is added and incubated for 2 hours. Then 10 ml of ethanol is added to stop the reaction before titrating with 0.05N NaOH to pH 9.5. The titration should be carried out with a glass electrode and the incubation mixture plus alcohol should be stirred with a magnetic bar.

A substrate control (20 ml half & half plus 10 ml water) is run to correct for the amount of alkali required to neutralize the salts present in the cream.

An enzyme control is also run to correct for the amount of alkali required to neutralize the salts present in the enzyme.

The increase in acidity corresponding to a 1% hydrolysis of the butterfat is equivalent to a titration difference of 1.65 ml and 0.05N NaOH. Activity is calculated from the following relationship:

$$\text{CLU} = \frac{\text{0.05N NaOH produced in 20ml Substrate}}{\text{g Enzyme used}}$$

The term "CLU" refers to cream lipase units.

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Effect of pH, Temperature and Time on Lipase Activity

The effects of temperature and pH on tributryin hydrolysis by the present lipase is noted in Figs. 2-4. As seen in Figure 2, the optimum temperature for enzyme activity is approximately 42.5°C, while Figure 3 shows that the optimum pH is close to 7.0. A sharp decrease in activity as the temperature is raised can be seen in Figure 4, the effect being more dramatic with an increase in pH. The plots given in Fig. 5a-f also indicate that an increase in time accentuates the adverse effect of pH values above 6.2, although pH 7.0 is clearly optimal at temperatures below 60°C.

Triglyceride Hydrolysis

The present lipase has been used to hydrolyze specific triglycerides (tricaprylin, tricaproin, and tributyrin) into their respective free fatty acids (FFAs) (caprylic, caproic, and butyric acids. Conventional gas chromatography techniques can be used to measure these FFAs.

Hydrolysis has been measured in the following manner. Five gm of triglyceride is blended with 100 ml phosphate buffer (pH 6.5, 0.05 m) containing 1% gum arabic to form an emulsion, which is shaken with 2.0 ml of fungal lipase (100 mg) for 4 hours at 33°C. To stop the reactions, 10 ml of ethanol is added, and the resulting free fatty acids are measured by gas chromatography.

The free triglyceride hydrolysis results are as follows:

Table 2

Enzyme Treated Substrate	% Free Fatty Acid	Ratio $(C\Delta)/C_4$
Tributyrin (C4)	3.39	1.00
Tricaproin (C6)	3.76	1.11
Tricaprylin (C8)	5.26	1.55

The present lipase hydrolyzes both tricaproin and tricaprylin more efficiently than it hydrolyses tributyrin. This type of hydrolysis can be demonstrated by taking a ratio of the two numbers (C/C_4). Increased rate of hydrolysis can be shown by the ratio TC/TB. Typically, the increased rate of activity results in equivalent C_8/C_4 production. This specificity is unique for the present lipase.

The following table shows the tricaprylin (TC) to tributyrin (TB) hydrolysis ratio (TC/TB) for known lipases, as cited by known authors:

TABLE 3

RELATIVE ACTIVITY OF LIPASE PREPARATIONS AS CITED IN LITERATURE

Preparation	Activity vs. Tributyrin	Activity vs. Tricaprylin	TC/TB	Cite
Hog pancreas	100	37	0.37	Enzymologia
Human milk	100	59	0.57	11 178 (1944)
Bovine pancreas	100	41	0.41	

Release of FFA from Milk Fat

	mol % C_4	mol % C_8		
Milk Lipase	13.9	1.8	0.13	Nelson J.H.
Steapsin	10.7	1.5	0.14	J Amer Oil Chem Soc
Pancreatic Lipase	14.4	1.4	0.09	49 559
Calf Esterase	35.0	1.3	0.04	1972
Esterase pancreatin	15.83	3.0	0.19	
Calf pregastric esterase	40.8	8.6	0.21	Farnham
Kid pregastric esterase	50.0	8.4	0.17	et al
Lamb pregastric esterase	44.8	8.3	0.19	1956

% of Total Free Fatty Acids (microequivalent basis) after 3 hrs. at 35°C on milk fat

Imported crude kid rennet paste	32.8	7.1	0.22	Harper W.J.
Domestic purified calf rennet paste	10.7	trace	.00	J. Dairy Sci
Calf oral lipase	36.7	4.8	0.13	40 556 (1957)
Kid oral lipase	44.4	7.6	0.17	
Lamb oral lipase	48.1	14.2	0.30	
Aspergillus lipase	43.1	20.2	0.47	
Milk lipase	13.5	10.2	0.76	
Pancreatic lipase	8.4	trace	.00	

u moles FFA liberated/ml enzyme

Syncephalastrum racemosum	11.2	7.6	0.68	Chopra et al J. Dairy SC 65 1890 (1982)

ml 0.1N Acid Produced in 2 hrs. 30°C

Penicillum roqueforti	1.45	0.56	0.39	
Aspergillus niger	1.20	2.18	1.82	Shipe, W.F. Arch. Bioch. 30 165 (1951)

Figures 6 and 7 illustrate the significantly better TC hydrolysis versus TB hydrolysis of the present lipase and how it can be increased with temperature. At 40°C and pH 7.0 the TC/TB ratio is

1.73, while at 50°C and pH 7.0 it is an even greater 2.49.

Milk Triglyceride Hydrolysis

The present lipase has been tested for hydrolysis of those triglycerides present naturally in milk. To test for hydrolysis, 180 ml of 10.5% butter fat cream is treated with 0.36 g (0.2%) and 0.90 g (0.5%) of either the present lipase or calf lipase. Samples are held for 15 days at 0-3°C before free fatty acid (C4 to C12) is determined by gas chromatography. A control cream sample without enzyme is carried also through the experiment.

The results of the milk test are as follows.

TABLE 4

Mg FFA/ml cream

Specific FFA	Control	0.2% Present Lipase	0.5% Present Lipase	0.2% Calf Lipase	0.5% Calf Lipase
C4	0.18	0.37	0.52	0.38	0.53
C6	0.09	0.32	0.43	0.13	0.20
C8	0.10	0.20	0.27	0.12	0.15
C10	0.14	0.30	0.41	0.20	0.16
C12	<0.05	<0.05	0.09	0.05	0.05

TABLE 5

% Hydrolysis of Specific Glyceride

Specific FFA	Control	0.2% Present Lipase	0.5% Present Lipase	0.2% Calf Lipase	0.5% Calf Lipase
C4	6.0	12.3	17.3	12.7	17.7
C6	4.5	16.0	21.5	6.5	10.0
C8	11.9	23.8	32.1	14.5	17.8
C10	8.3	17.9	24.4	11.9	9.5
C12	<0.8	<0.8	1.5	0.8	0.8

TABLE 6

% Hydrolysis of Specific Glyceride
(Corrected for FFA present in Control)

Specific FFA	0.2% Present Lipase	0.5% Present Lipase	0.2% Calf Lipase	0.5% Calf Lipase
C4	6.3	11.3	6.7	11.7
C6	11.5	17.0	2.0	6.0
C8	11.9	20.2	2.6	5.9
C10	9.6	16.1	3.6	1.2
C12	0	(0.7)	0	0

Although FFA production of the present lipase is nearly identical to that of calf lipase for C4 production, all of the other FFA data show that the present lipase hydrolyzes the glycerides of C6, and C8, and C10 to a much greater extent. For example the present lipase shows, respectively, 5.8x, 4.6x, 2.7x more C6, C8, C10 than the calf lipase at 0.20% concentration, and 2.8x, 3.4x and 13.4x at 0.50% concentration.

Triglyceride Hydrolysis in Oils

The present lipase has been tested also for production of 5% emulsions of soy, butter, and coconut oils. Chromatography of the treated emulsions (4 hours

at 33°C) reveals the ability of this lipase to hydrolyze a whole range of triglycerides. The level of certain triglycerides in the mixtures and the ability to fully emulsify the substrates can alter the rate of specific free fatty acid released as illustrated in Table 7.

Fatty Acid Determined	Soy Oil % FFA	Butter Oil % FFA	Coconut Oil % FFA
C2	**ND	ND	ND
C4	ND	0.16	ND
C6	ND	0.066	0.029
C8	ND	0.014	0.19
C10	ND	0.062	0.042
C12	ND	0.19	0.22
C14	ND	0.062	0.047
C16			
C18	0.13	0.57	0.57
C18-1	0.97	0.072	0.072
C18-2	3.25	0.24	1.61

**ND = none detected

Cheese Preparation

Cheese has been made with the present lipase in the following manner. Milk is obtained from a diary farm, batch pasteurized at 145°F for 30 minutes, and then held at 36°F until the following day. (Colby cheese is made using Hansen CH 60Y™ as a starter). Equivalent activity amounts of the present lipase and Miles 600 calf lipase (by cream assay) are preweighed and mixed with salt to give 2% salt by weight. Eight treatments of the cheese are made using 20 pound blocks. Control blocks with salt but without enzyme are made as well.

The resulting cheeses are sampled at 1, 28, 72, 102, and 198 days. They are evaluated organoleptically and analyzed for free fatty acids by gas chromatography, known acid degree value (ADV) tests, and also by 12 % TCA soluble nitrogen tests.

After 72 hours, the cheeses having 0.048% present lipase and 0.128% calf lipase have about the same ADV and total FFA, which is 50-70% greater than that of the control. However, at 102 and 198 days, the concentrations of enzyme used (and for which data has been tabulated) are too high for good flavor in that too much free fatty acid has developed. The calf lipase produces too much butyric acid, and the present lipase, too much total free fatty acid C14-C18.

At lower concentrations (0.012 and 0.024%) the present lipase gives good aged flavor without rancidity associated with butyric acid at 102 days. The control has weak flavor at this point, while the calf lipase showed the typical rancidity associated with butyric acid at days, even with low levels of the enzyme preparation (0.064 and 0.032%).

Figs. 8 and 9 illustrates the triglyceride hydrolysis data for the control cheese and cheeses prepared with 0.048% NM 313 lipase (present lipase) and 0.128% calf lipase. It is readily apparent that at 72 days and 102 days, the butyric acid content in cheese made from the calf lipase is very elevated over that of the control. On the other hand, cheese made with the present lipase is more like the control cheese as far as butyric acid levels.

The present lipase reduces the mild cheese aging process by 2-5 times. At 102 days the present lipase-added cheese shows greater C16, C18, C18-1 and

C18-2 content than the control cheese, indicating an acceleration (i.e. more rapid flavor development) of the usual ripening process. Thus, cheeses manufactured with the present lipase develop a Cheddar like flavor while those of the calf lipase produces an Italian type flavor. It is apparent that the lipase derived from the present Aspergillus organism is unique in shortening the storage time needed for Cheddar and other mild flavored cheeses.

Having described the invention with particular reference to preferred form, it will be obvious to those skilled in the art to which the invention pertain, that, after understanding the invention, various changes and modifications may be made without departing from the spirit and scope of the invention as defined by the appended claims.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A biologically pure culture of a variant of Aspergillus species known as ATCC No. 20719 as hereinbefore defined which produces a lipolytic enzyme having a tricaprylin to tributyrin hydrolysis ratio greater than 0.75.

2. A culture of claim 1 wherein the enzyme produced has a ratio greater than 1.5.

3. A lipolytic enzyme derived from an organism belonging to the Aspergillus species known as ATCC No. 20719 as hereinbefore defined which has a tricaprylin to tributyrin hydrolysis ratio of at least 0.75.

4. An improved method of producing mild cheeses with characteristically low butryic acid content, the improvement comprising adding to the cheese curd or milk an effective amount of lipolytic enzyme according to claim 3 having a tricaprylin to tributyrin hydrolysis ratio of at least 0.75, whereby the aging of the cheese is accelerated.

5. The method of claim 4 wherein the mild cheese is cheddar cheese.

6. The method of claim 4 wherein protease is added in an amount effective to soften the cheese.

7. An improved method of producing cheese, the improvement comprising adding to the cheese curd or milk an

effective amount of a first lipolytic enzyme according to claim 3 having a tricaprylin to tributyrin hydrolysis ratio of at least 0.75 and a second lipolytic enzyme with a tricaprylin to tributyrin hydrolysis ratio of less than 0.75.

8. The method of claim 7 wherein a protease is added in an amount effective to soften the cheese.

9. A biologically pure culture according to claim 1 substantially as hereinbefore described with reference to any one of the examples.

10 10. A lipolytic enzyme according to claim 3 substantially as hereinbefore described with reference to any one of the examples.

11. A method according to claim 4 substantially as hereinbefore described with reference to any one of the examples.

DATED: 11 January 1990
PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:
GENENCOR INC.

EFFECT OF MYCELIUM CONCENTRATION
ON RELEASE OF LIPASE FROM MYCELIUM

Fig. 1

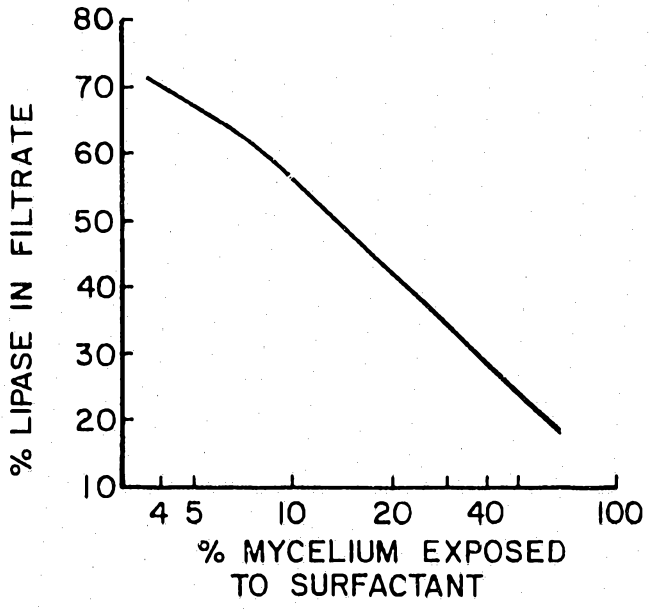
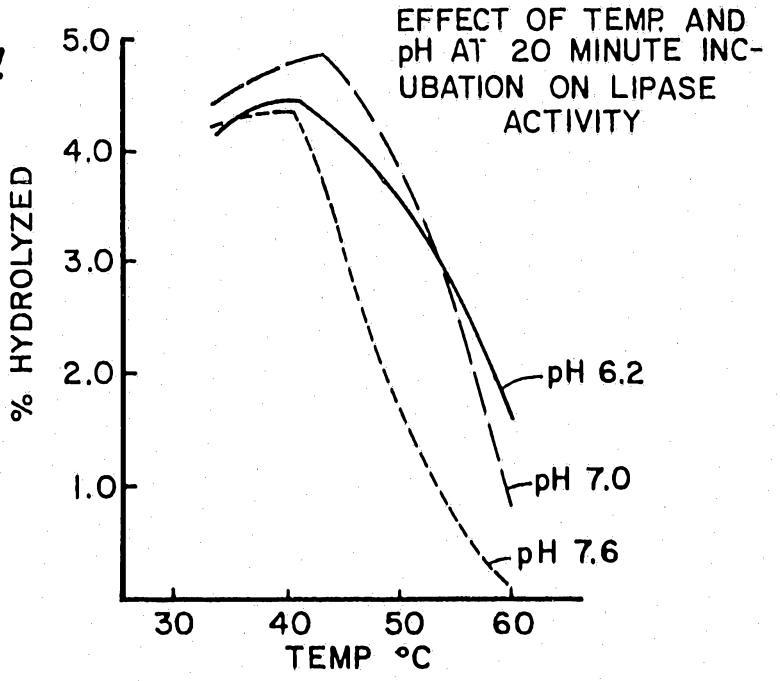


Fig. 4



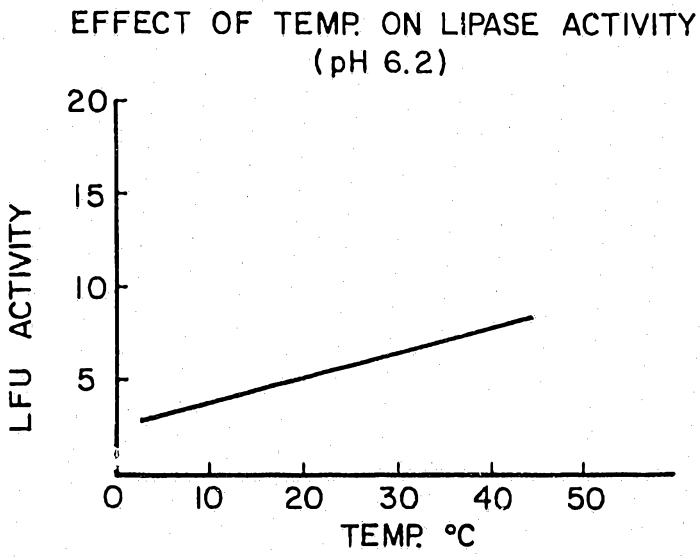

EFFECT OF TEMP. ON LIPASE ACTIVITY
(pH 6.2)
LFU ACTIVITY
20
15
10
5
0
10
20
30
40
50
TEMP. °C

Fig. 2

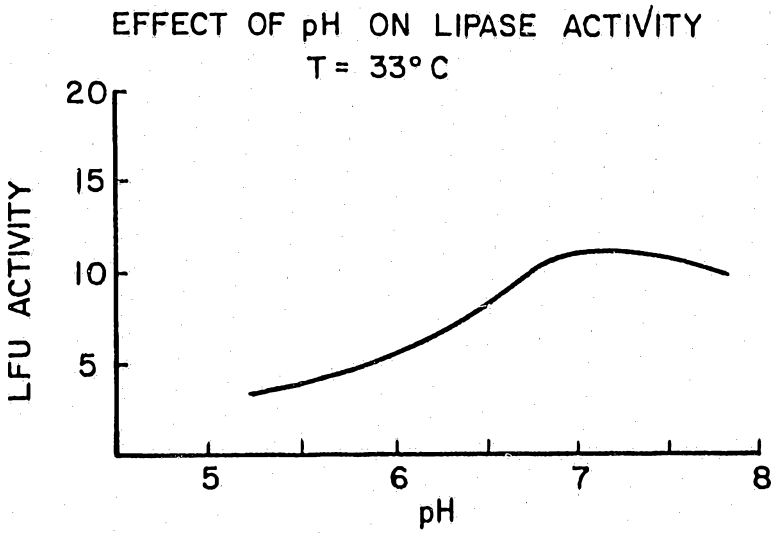

EFFECT OF pH ON LIPASE ACTIVITY
T = 33° C
LFU ACTIVITY
20
15
10
5
5
6
7
8
pH

Fig. 3

EFFECT OF TIME ON LIPASE ACTIVITY
AT 33° C — 40° C — 42.5° C

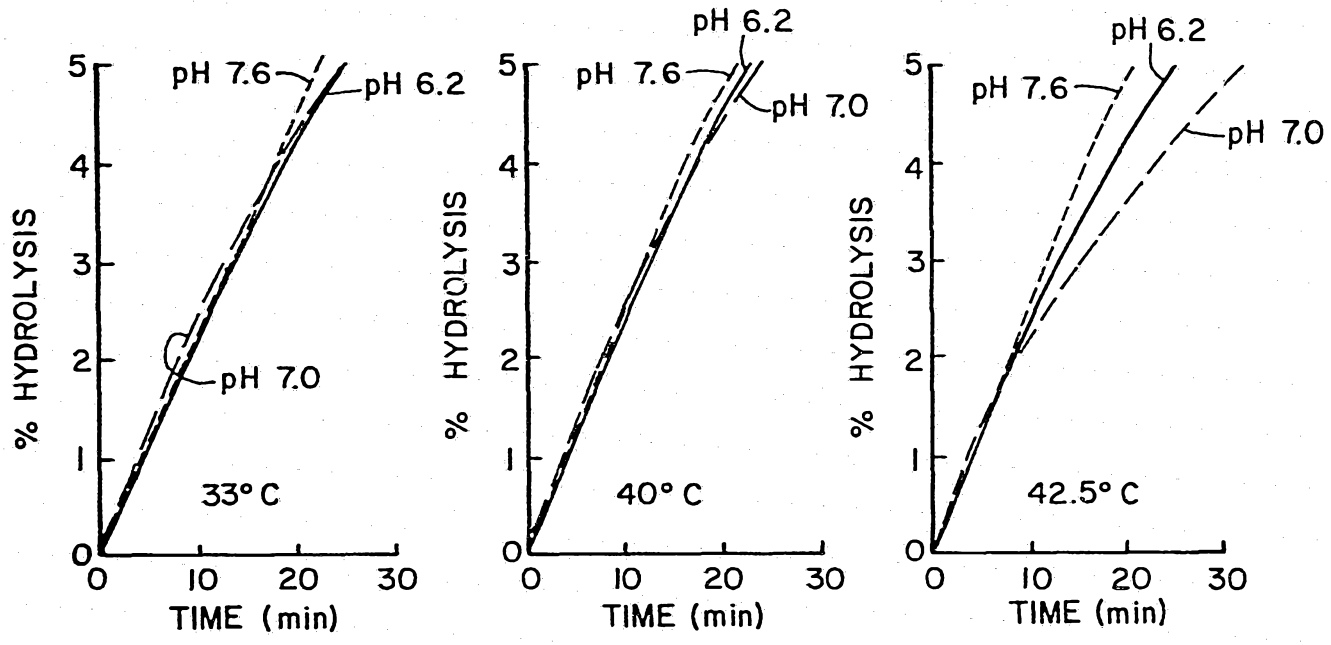


Fig. 5a

Fig. 5b

Fig. 5c

EFFECT OF TIME ON LIPASE ACTIVITY
AT 45°C —50°C—60°C

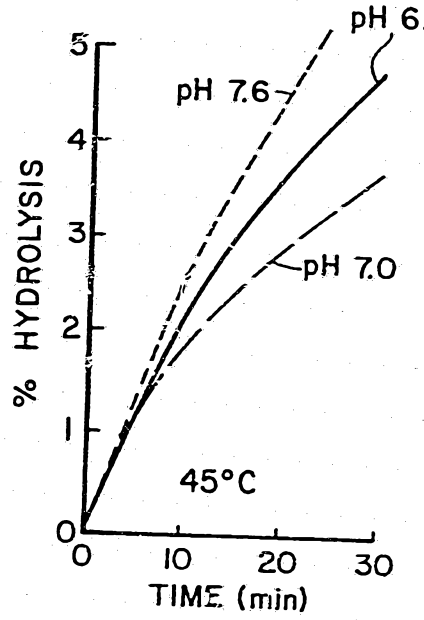


Fig. 5d

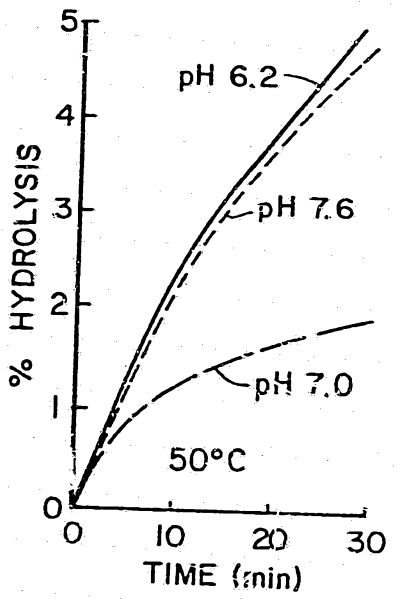


Fig. 5e

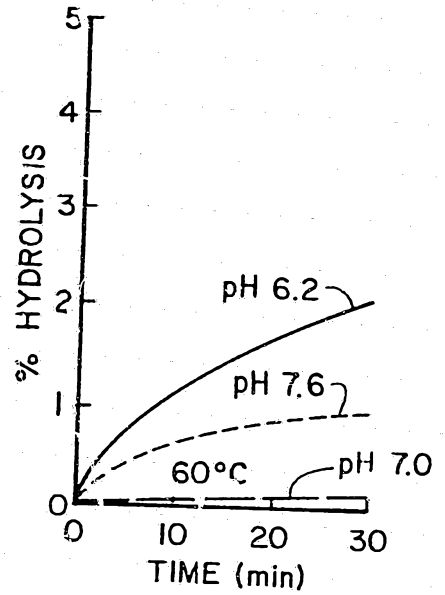


Fig. 5f

EFFECT OF TIME ON TRICAPRYLIN HYDROLYSIS

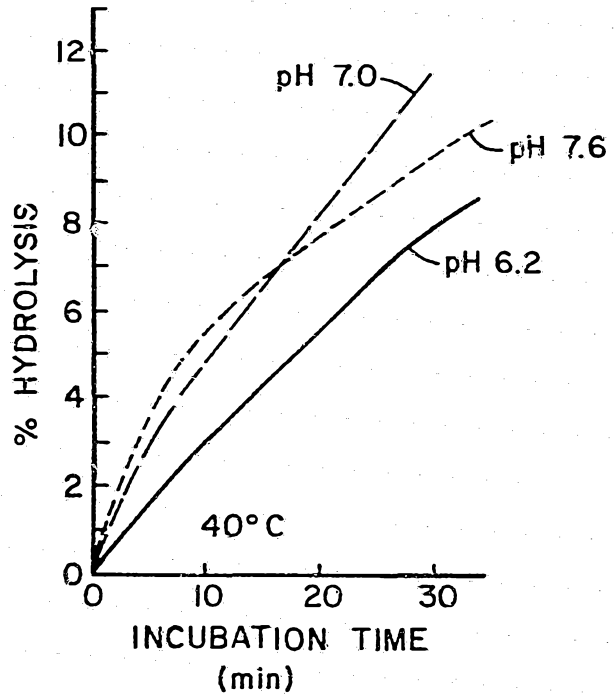


Fig. 6

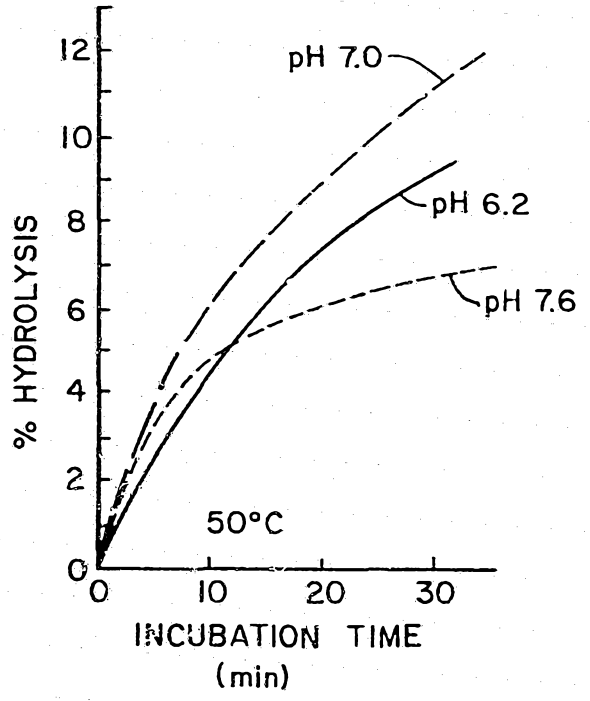


Fig. 7

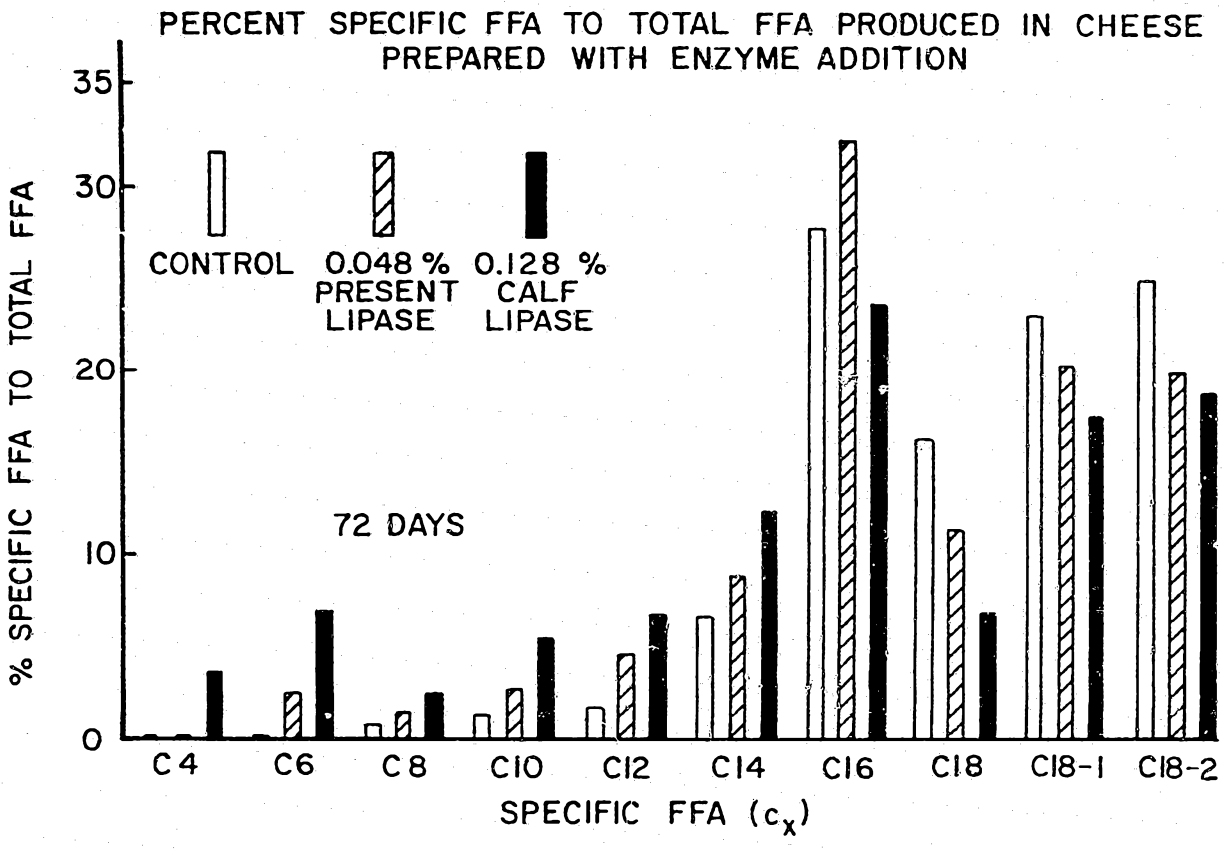

PERCENT SPECIFIC FFA TO TOTAL FFA PRODUCED IN CHEESE PREPARED WITH ENZYME ADDITION
% SPECIFIC FFA TO TOTAL FFA
35
30
20
10
0
CONTROL
0.048 % PRESENT LIPASE
0.128 % CALF LIPASE
72 DAYS
C4
C6
C8
C10
C12
C14
C16
C18
C18-1
C18-2
SPECIFIC FFA (c_x)

Fig. 8

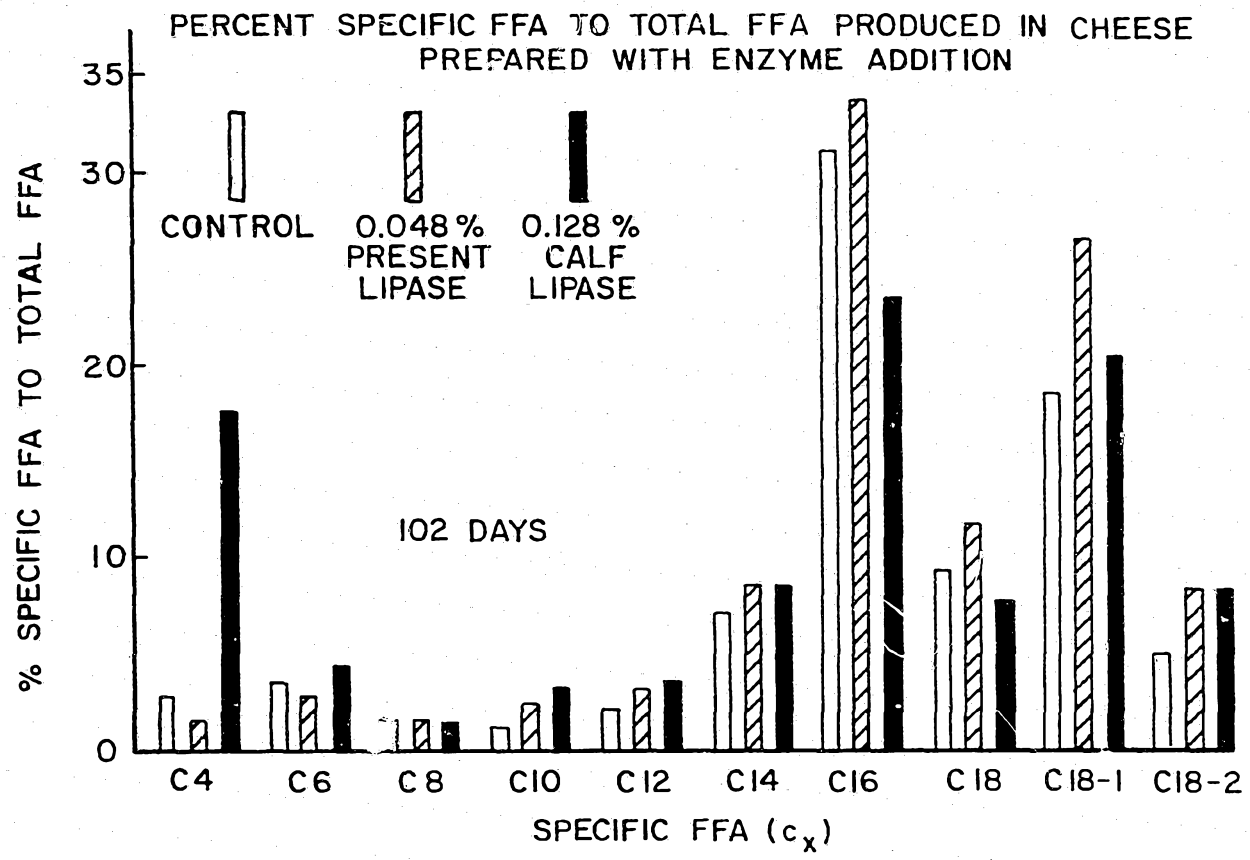


Fig. 9