decreases in that order for those series, in contrast to the observation above for the homologous series of methyl esters, and the negative correlation between density and cetane number in ASTM D-976. When this equation is used to calculate the cetane indexes of sets of esters having the same carbon numbers using the boiling points and densities from Table II, we find cetane indexes which are essentially the same for all members of the set. For example, for the esters with 13 carbon atoms, the calculated cetane indexes were 43.24, 43.61 and 43.84 for methyl laurate, 2-propyl decanoate and 1-propyl decanoate respectively. This occurs because the increase in cetane number which is predicted to accompany an increase in boiling point is almost exactly offset by the decrease in cetane number predicted to accompany an increase in density.

These results demonstrate that, although ASTM D-976 may be useful in predicting trends in cetane numbers for a homologous series of fatty acid esters, it is not able to predict variations in cetane numbers when more complex structural variations are being considered. Perhaps physical properties other than just boiling point and density, for example surface tension and heat capacity, need to be considered before accurate cetane numbers may be calculated for fatty acid esters.

Cetane numbers for the methyl esters of the saturated fatty acids varied in a non-linear manner with chain length of the fatty acid. Cetane numbers also increased with increasing molecular weight for esters of the normal alcohols when fatty acid was kept constant. The cetane number increase resulting from an increase in the molecular weight of the alcohol of the ester was less than that for the same increase in molecular weight of the fatty acid portion of the ester.

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Purification and Properties of the Lipase from *Candida deformans* (Zach) Langeron and Guerra

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ABSTRACT

Palm oil being solid at room temperature could be converted into a fluid oil by substitution of about 40-50% of its palmitic acid. This could be achieved by a fermentation process or using yeast lipase.

Candida deformans CBS 2071 seemed suitable for this purpose; therefore, its lipase was isolated and studied. This enzyme was purified by acetone precipitation followed by chromatographies on Sephadex C 50 and Sephadex G 150. The purification factor achieved was ×70, and the protein and activity yields were 0.25% and 18%, respectively. The homogeneity of the purified enzyme was verified by polyacrylamide gel electrophoresis. The enzyme molecular weight was estimated at 207,000. Its activity is optimal between 40 C and 50 C and its optimum pH is 7.0. This enzyme is thermoresistant and loses only 14% and 18% of its activity, respectively, when heated to 45 C and 55 C for 30 min. Its activation energy was 2.75 kcal.mole^{-1} and its inactivation energy was around 21 kcal.mole^{-1}. This enzyme is activated by Ca^{++}, Mg^{++} and Co^{++} and inhibited by Cu^{++}, Zn^{++}, and p-chloromercuribenzoate (pCMB) and EDTA.

The synthesis of this lipase is induced by lipid substrates in the culture medium and inhibited by glucose. This enzyme attacks primarily the 1- (or 3-) position of all triglycerides tested. Hydrolysis was preferential for triglycerides containing short chain fatty acids. The triglycerides with monounsaturated monoacids were more quickly hydrolyzed than those with saturated monoacids. The presence of two and especially three double bonds in the fatty acid chain seemed to slow down the rate of hydrolysis.

INTRODUCTION

Numerous papers relative to lipase-producing microorganisms have been published, and most of them are concerned with selection and identification of strains, growth characteristics, lipase production mechanisms and, in some cases, with the purification and enzymatic properties of lipases. These microbial lipases have received much attention because of their potential use in industry. Seitz (1) and Macrae (2) reported some interesting new applications for microbial lipases, developed particularly in the oils, fats and dairy industries, to make foods palatable and acceptable. For the purpose of biofashioning palm oil, it may be possible to exploit the potential of some microorganisms.

Crude palm oil is solid at room temperature because of

the fact that it contains a large percentage of palmitic acid (45-52%). This state obviously is a considerable barrier to its use as table or cooking oil. The oil is often fractionated by crystallization, which leads to a fluid fraction or olein (60% of the crude oil) and a solid fraction or stearin (40%). The fluid fraction can then be used for cooking and seasoning after refining. The solid fraction has a lower market value, although it is exported in part or used locally for the manufacture of margarine, vanaspati and oleochemicals.

Regarding the glyceride structure of palm oil, Faulkner et al. (3) observed that about 90% of the palmitic acid is on the external position of the glycerol. According to this observation, we propose a microbiological method for the substitution of these undesirable fatty acids using a continuous fermentation process or a process involving the immobilization of enzyme in order to eliminate 40-50% of the palmitic acid. Therefore, the yeast strain used or its lipase must have a high specific activity for the 1-3 positions in order to produce a high level of diglycerides 1-2 and few monoglycerides. The diglycerides produced may then be esterified using suitable fatty acids.

Martinet (4) remarked that the *Candida deformans* CBS 2071 strain preferentially hydrolyzes in culture media the fatty acids in the 1-3 positions of triglycerides. This yeast strain seems thus to have the appropriate enzyme system for our purposes. To check this, we purified and studied the enzymatic properties of the extracellular lipase of *Candida deformans* CBS 2071. Besides palm oil or its fractions, we also used rapeseed oil and pure homogeneous triglycerides.

MATERIALS AND METHODS

Biological Material

The yeast strain used was *Candida deformans* (Zach) Langeron and Guerra CBS 2071, obtained from the Centraal Bureau Voor Schimmelcultures (CBS), Yeast Division, Delft, The Netherlands.

According to Meyer et al. (5), Yarrow has observed crossing between *Candida deformans* and several strains of *Saccharomycopsis lipolytica.* It is therefore possible that those two strains belong to the same species. However, the *Candida deformans* strain is still admitted as a separate species in "The Yeast—A Taxonomic Study, 1984" (6).

Culture Techniques

The *Candida deformans* culture was maintained at 4 C on agar (3.0%) slants containing Difco yeast extract (0.5%) and glucose (0.5%). Cultures in liquid media were performed in Erlenmeyer flasks filled to 10% of their volume, incubated at 28 C and shaken (80 oscillations/min; amplitude 7.5 cm). The media pH was maintained at 6.5 with phosphate buffer (0.2 M). Yeast Nitrogen Base (YNB-Difco) was used as nitrogen source. This solution was sterilized by filtration (Millipore filter 0.45 μm) and added to the medium (0.67% final concentration).

Rapeseed oil or a solid fraction of palm oil was used as a carbon source (0.5%) and was sterilized by autoclaving at 110 C for 30 min. When a solid fraction of palm oil was used as a carbon source, an emulsifier (Polyoxyethylene glycol palmito-stearate - PEG 600) was added to the medium at 0.05%.

Growth Curve and Dry Matter Determinations

The cells were washed with ethanol and hexane on a filter (Swinnex Millipore 0.45 μm) and resuspended in distilled water by ultra-sonic treatment for 15 to 20 sec.

Cell growth was monitored by measuring the suspension optical density with a Klett Summerson colorimeter (blue filter: 400 to 450 nm). The relationship between O.D. and dry matter thus could be established.

Enzyme Extract Preparation

The cells were harvested at the end of log phase by centrifugation at 16,000 × g for 10 min. Cold acetone (4 C) was added slowly to the supernatant (60%, v/v) and the mixture was kept at 4 C for 30 min. The mixture was then centrifuged at 16,000 × g for 10 min. The centrifugate was redispersed in tris maleate buffer (2.5 mM; pH 7.0). This protein solution was then centrifuged at 105,000 × g for 1 hr. The resulting supernatant constituted the enzyme extract preparation.

Enzyme Assay

The enzyme assay was performed in a reaction mixture according to the method of Menassa (7) at 45 C and pH 7.0 for 2 hr. The PVA substrate emulsion was prepared according to the method of Biehn et al. (8).

In the case of palm oil or its stearin fraction or solid triglyceride standards, the product is melted and heated to 80 C and then added in the hot PVA solution. After emulsification the mixture is allowed to cool to room temperature.

The reaction was stopped by the addition of 40 ml of an alcohol:acetone (1:1) mixture.

Assay of the Reaction Products

Lipids extraction. The reaction mixture was transferred into a 250 ml separatory funnel and acidified with 10 ml HCl (4N). The lipids were extracted three times with a total of 100 ml hexane. The combined organic phase was washed until neutrality with distilled water and filtered through glass wool containing anhydrous Na_2SO_4. Next, the solvent was eliminated under reduced pressure with a rotary evaporator. The residue was desiccated under nitrogen atmosphere until constant weight. The lipids then were dissolved in a known volume of chloroform to obtain an approximate 1% (w/v) solution.

Lipids assay. The assay was performed by TLC using Silica gel 60 G as the stationary phase. The plate (20 × 20 cm) was prewashed with distilled diethyl ether in a saturated chamber. Aliquots of 3 to 5 μl were deposited automatically on the plate with a Camag-Linomat III apparatus. The migrating phase was n hexane:ethyl ether:acetic acid mixture (70:30:1). The plate was then dried at 120 C for 10 min and sprayed with a saturated aqueous copper acetate:pure phosphoric acid, 50:50, v/v. The spots on the plate were revealed by charring at 180 C for 15 min. For the study of homogeneous triglycerides with saturated fatty acids, we used plates (20 × 20 cm) prepared in the laboratory (30 g Silica gel 60 G/60 ml distilled water; thickness: 0.25 mm). The migrating solvent mixture was n hexane: ethyl ether:acetic acid (80:20:1). Then sulfochromic acid was sprayed on plates and the spots were revealed by charring at 180 C for 15 min. The spots revealed were quantified by photodensitometry with a Camag TLC Scanner at 500 nm, and the peaks were integrated by a Delsi Enica 10 calculator.

Protein Assay

Proteins were assayed by the method of Lowry et al. (9). Bovine serum albumin was used for establishing the standard curve.

Glucose Assay

Glucose in the culture supernatant was assayed by the method of Somogyi (10). Optical density was measured at 520 nm using a Beckmann spectrophotometer.

Estimation of Molecular Weight

The molecular weight of the purified lipase was determined by gel filtration (Sephadex G 150) as described by Andrews (11). Cross-linked bovine serum albumin was used as molecular weight standard (tetramer: 264,000; trimer: 198,000; dimer: 132,000; monomer: 66,000).

Electrophoresis

Electrophoresis was performed on a 12.5 × 4.5 cm plate of polyacrylamide gel (7.5%, w/v) soaked with tris glycine buffer (0.05 M; pH 8.9). Migration was obtained at 450 V for 3 hr. Proteins were fixed with trichloroacetic acid (12.5%, w/v) for one hr and stained with Coomassie blue (0.25%, w/v) overnight.

Results Expression

One unit of lipase activity was defined as that which produces 1 μmole of free fatty acid (FFA) under the conditions used. Then, specific activity was expressed as μmole FFA/mg protein/min.

RESULTS AND DISCUSSION

Localization of the Lipase

Lipase activities of *Candida deformans* in the filtered and concentrated culture medium supernatant and in the ground cell preparation were assayed. The extracellular activity was shown to represent 80% of total activity, and the lipids content in the enzyme preparation was shown not to influence the results obtained.

Purification of the Lipase

The purification of the lipase was performed according to the methods of Tomizuka et al. (12) and Ota et al. (13) with some modifications.

The specific activity of the supernatant culture containing 780 mg proteins was found to be 0.24 μmole FFA/mg/min. The enzyme extract obtained by precipitation of the supernatant proteins with cold acetone (60%, v/v) was concentrated in an Amicon cell (PM: 10,000 membrane) under nitrogen atmosphere. The resulting concentrated solution contained 39 mg proteins, and specific activity was close to 1.08 μmoles FFA/mg/min.

The precipitated proteins were purified by chromatography on SP Sephadex C 50 and Sephadex G 150 columns. The active fractions were gathered together and the amount of proteins was close to 5 and 2 mg, respectively. Specific activities were found to be 8.40 and 16.80 μmoles FFA/mg/min, respectively.

Electrophoresis

Following dialysis overnight against distilled water, 20 μl of the purified lipase containing 10 μg proteins was deposited on a filter paper (0.5 × 1 cm) and put onto the polyacrylamide gel. A single protein band was obtained after electrophoresis.

Properties of the Lipase

The lipase molecular weight was estimated at 207,000. Its activity is optimal between 40 C and 50 C and its optimum pH is 7.0. The activation energy was 2.75 kcal/mole and the inactivation energy was around 21 kcal/mole. The activity of the enzyme was stable at −18 C. The enzyme was observed to lose 12% and 50% of its activity after 25 days storage at 4 C and 27 C, respectively.

Enzyme effectors were diluted to 3 mM final concentration in tris maleate buffer (2.5 mM; pH 7.0). The enzyme reaction was performed at 45 C, pH 7.0, for 2 hr. The results are summarized in Table I. Cations were provided as chlorides or sulfates. The monovalent cations tested (K^+ and Na^+) did not significantly affect the enzyme activity. Among bivalent cations used, Ba^{++} did not affect the activity of the lipase, while Ca^{++}, Mg^{++} and Co^{++} were shown to be favorable to the action of this enzyme. On the other hand, Cu^{++}, Zn^{++} and Fe^{++} strongly inhibited the enzyme. Ni^{++} and Mn^{++} only slightly reduced the enzyme activity. A strong oxidation of lipids also was observed in the presence of Fe^{++}, Cu^{++}, and Co^{++} due to their catalytic properties for the oxidation of fats and oils. The inhibition in the presence of EDTA indicates that the enzyme is dependent on a metal cofactor in its active site. As iodoacetamide, N. ethylmaleimide and parachloromercuribenzoate (pCMB), which are classical binders of thiol groups, inhibited the enzyme, its active site must contain an -SH group. These results are similar to those obtained by Chandan and Shahani (14) on milk lipase.

TABLE I

Action of Some Effectors on Lipase Activity

Effectors	Activity*
Control	100
K^+	109
Na^+	91
Ca^{++}	129
Ba^{++}	99
Zn^{++}	58
Mn^{++}	84
Fe^{++}	64
Ni^{++}	90
Cu^{++}	15
Mg^{++}	122
Co^{++}	118
EDTA	57
Iodoacetamide	80
N - Ethylmaleimide	90
Parachloromercuribenzoate	24

*Activity expressed as % of control (without effector).

Study on the Regulation of the Biosynthesis of the Lipase

Activity of growing cells. Four carbon substrates (0.50%, w/v) were tested: rapeseed oil (*Primor* variety), glucose, ethanol and rapeseed oil (0.25%, w/v)-glucose (0.25%, w/v) mixture. The basal medium used was Yeast Nitrogen Base.

Samples were taken during the growing phase of cell culture. Proteins and enzyme activity assays were performed on the culture supernatant after filtration and concentration. It was shown that the lipids content of the supernatant did not affect the results of enzyme activity assays.

Lipase activity appeared during growth on rapeseed oil (Fig. 1). This activity along with protein content decreased sharply at the beginning of the stationary phase. No lipase activity was detected during growth on glucose and ethanol. The biosynthesis of the enzyme thus was induced by the presence of rapeseed oil.

Cell growth on the rapeseed oil-glucose mixture revealed that glucose inhibited the biosynthesis of lipase. In this experiment, lipase activity appeared only when glucose was used up (Fig. 2). Beyond 15 hr culture time, lipase activity and protein content decreased slowly.

The results concerning the activity of growing cells are summarized in Figure 3, where activity is plotted versus

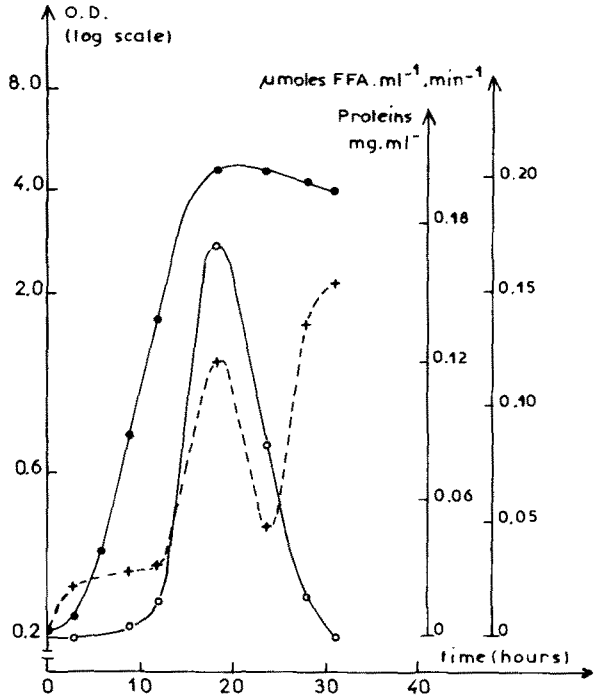

FIG. 1. Activity of growing cells on rapeseed oil (0.50%, w/v). •—• growth curve; +---+ proteins (mg.ml^{-1}), and ○—○ lipase activity (µmole FFA.ml^{-1}.min.$^{-1}$).

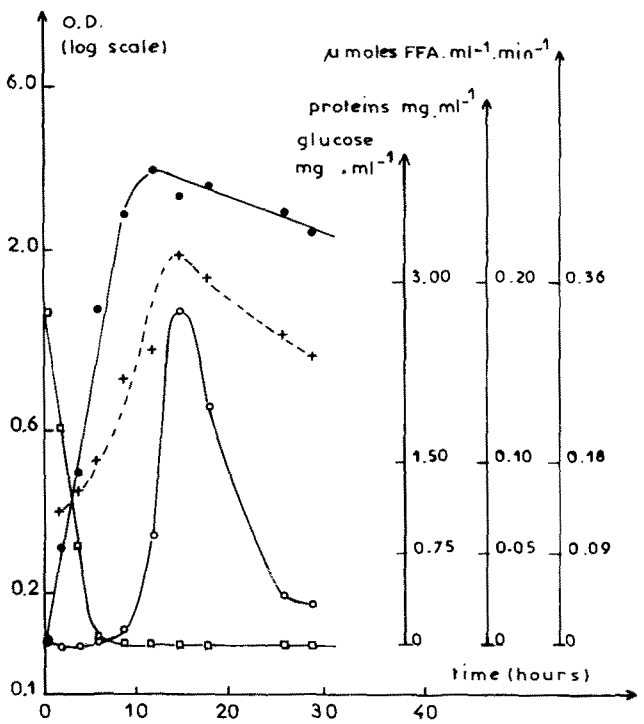

FIG. 2. Activity of growing cells on rapeseed oil (0.25%, w/v)-glucose (0.25%, w/v) mixture. •—• growth curve; +---+ proteins (mg.ml^{-1}); ○—○ lipase activity (µmole FFA.ml^{-1}.min.$^{-1}$), and □—□ glucose (mg.ml^{-1}).

growth expressed as mg dry matter per ml (the arrow indicates when glucose was used up). These results are similar to those reported previously. Dherbomez et al. (15) showed that addition of lipids to the culture medium stimulated the production of lipase by *Candida lipolytica*. Tsujisaka et al. (16) reported that the *Geotrichum candidum* LINK strain

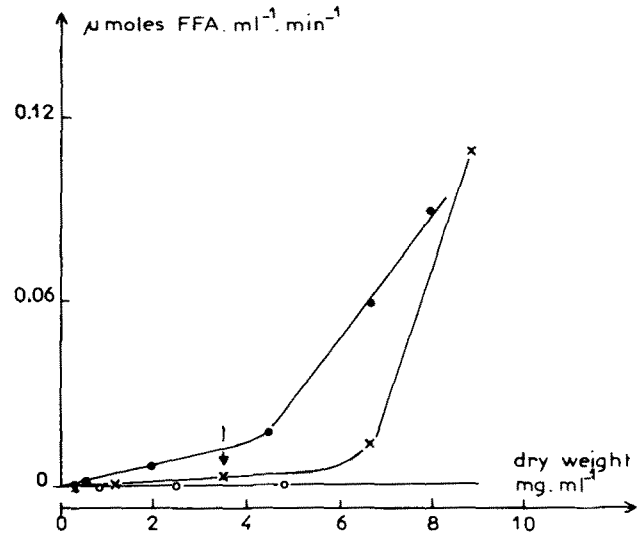

FIG. 3. Activity of growing cells versus dry matter content during growth on rapeseed oil (•), rapeseed oil + glucose (×) and glucose or ethanol (○). The arrow indicates the end of glucose uptake in the case of the rapeseed oil + glucose mixture.

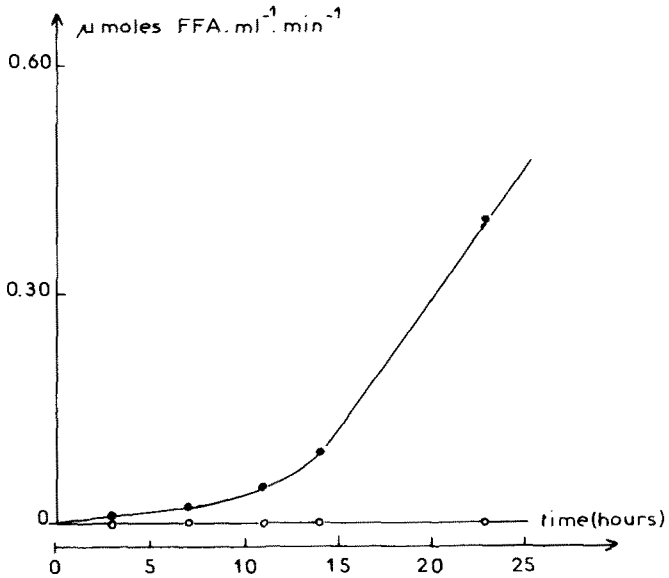

FIG. 4. Activity of the non-growing cells versus time. (•) rapeseed oil, and (○) glucose or glucose + rapeseed oil.

secreted lipase only in the presence of a specific substrate (oils, fatty acids) in the culture medium. Similarly, Imamura and Kataoka (17) found that the production of lipase by *Penicillium roquefortii* was inhibited by the presence of sugars (lactose, glucose or galactose) and was activated by the addition of a lipid substrate in the growth medium.

Activity of non-growing cells. Cells were grown on glucose (0.50%, w/v) and YNB (0.67%, w/v). After 24 hr culture, the cells were harvested and washed with sterile distilled water by centrifugation at 16,000 × g for 10 min and dispersed in sterile phosphate buffer 0.2 M pH 6.5. The suspension was divided into 3 portions and put into contact with rapeseed oil (0.50%, w/v), glucose (0.50%, w/v) and glucose (0.25%, w/v)-rapeseed oil (0.25%, w/v) mixture. The mixtures did not contain any nitrogen source and could not support cell growth.

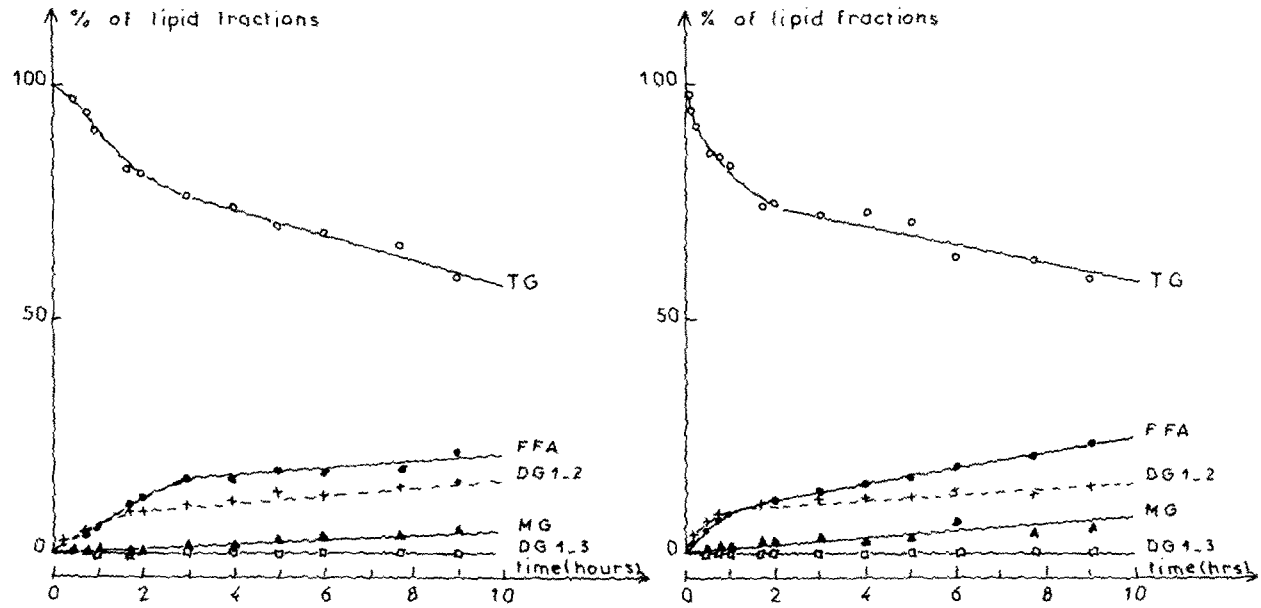

FIG. 5. Hydrolysis kinetics of glycerides. (○) triglycerides (TG); (●) free fatty acids (FFA); (+) diglycerides 1-2 (DG 1-2); (▲) monoglycerides (MG), and (□) diglycerides 1-3 (DG 1-3).

TABLE II

Influence of the Nature of the Fatty Acid on the Rate of Hydrolysis of Triglycerides

Saturated fatty acids	Specific activity	Monounsaturated fatty acids	Specific activity	C 18 fatty acids	Specific activity
C 12:0	15			C 18:0	3.5
C 14:0	10	C 14:1	19	C 18:1	6.5
C 16:0	4	C 16:1	11	C 18:2	3
C 18:0	3.5	C 18:1	6	C 18:3	1.5

Samples were taken at regular time intervals and centrifuged. The supernatants were filtered and concentrated. Total protein and enzyme activity assays were performed.

No activity was observed after 23 hr contact of non-growing cells with glucose or glucose-rapeseed oil mixture. On the other hand, in the presence of rapeseed oil alone, a progressive increase of activity was shown versus time (Fig. 4). This confirms the induction effect by rapeseed oil and the inhibition by glucose of the biosynthesis of the lipase from *Candida deformans*.

Study of the Specificity of the Lipase

Hydrolysis kinetics of glycerides. Rapeseed oil and solid fraction of palm oil glycerides were hydrolyzed. Figures 5a and b show this hydrolysis lasted 9 hr and produced diglycerides 1-2 (DG 1-2) and free fatty acids (FFA). There was no formation or accumulation of diglycerides 1-3 (DG 1-3). Monoglycerides appeared late, when diglyceride 1-2 content was high and that of triglycerides reduced. These results indicate that the lipase from *Candida deformans* CBS 2071 is specific for the primary hydroxyl of the glycerides. This enzyme is thus 1-3 specific and has a lipolytic activity similar to that of pancreas lipase (18), of *Rhizopus arrhizus* (19) and of different *Alcaligenes* strains (20).

Influence of the nature of fatty acids on the rate of hydrolysis. The hydrolysis kinetics of triglycerides with homogeneous fatty acids were determined. In the case of triglycerides with unsaturated monoacids, the reaction was carried out under nitrogen atmosphere and in the presence of BHT (butylhydroxytoluene) (1%, w/v) in the oil in order to limit lipid oxidation.

The results are summarized in Table II. According to this table, it appeared that: (a) the shorter saturated or unsaturated fatty acids are, the greater the rate of hydrolysis is; (b) triglycerides with monounsaturated fatty acids were more quickly hydrolyzed than those with saturated fatty acids, and (c) triglycerides with C 18:2 and especially with C 18:3 were hydrolyzed at a slower rate than those with C 18:0 and C 18:1. Multiple double bonds seemed to slow down the rate of hydrolysis.

These results agree with those of Ota et al. (21) and Hassing (22), who reported that triolein was more rapidly hydrolyzed than tristearin by the lipases of *Candida paralipolytica* and of *Corynebacterium acnes*, respectively. However, the lipase from *Candida deformans* studied here is different from pancreatic lipase (23), which is specific for long chain fatty acids. These results also agree with those described for *Saccharomycopsis lipolytica* by Ota et al. (24). This analogy could be considered as an additional argument to state that *Candida deformans* and *Saccharomycopsis lipolytica* should be considered in fact as a single species.

In conclusion, we can say that *Candida deformans* CBS 2071 or its lipase are suitable for our target. However, we should aim better the hydrolysis of 50% of the external fatty acids than 50% of palmitic acid, since we showed that practically only 1-2 diglycerides occur after the lipolysis and, according to the analysis of Faulkner et al. (3), we can therefore establish that after an hydrolysis of 50% of external fatty acids (33.33% of total fatty acids), palm oil

will be converted entirely into 1-2 diglycerides. This involves the loss of about 40% of the total palmitic acid and about 6% of the total oleic acid.

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♣Factors Affecting Cloud Point Analysis of Palm Oleins

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ABSTRACT

Some of the factors affecting cloud point determination of palm oleins are described. These are the type of container used, method of stirring, rate of stirring and bath temperature. The repeatability and reproducibility standard deviations of the method are determined from collaborative trials. Recommendations for the test are made to reduce the large variations among laboratories.

INTRODUCTION

Cloud point determination of palm olein is an important test in the palm oil industry. It provides an indication of the unsaturation of the sample and of the stability of the sample to crystallization at room temperature. The test forms part of the required specifications of the customer.

When a sample of melted fat is steadily cooled and agitated to low enough temperature, turbidity is induced due to the formation of fat crystals. Cooling is continued until there are sufficient crystals to form an opaque cloud (defined as the point when the thermometer in the sample is no longer visible). The temperature at which this opaque cloud is observed is designated as the cloud point.

When testing samples of unknown cloud point, the cooling bath should be started at a sufficiently high temperature to avoid supercooling and the temperature lowered slowly, keeping a gradient of 2-5 C between the bath and the sample. In the AOCS Methods Cc 6-25, a second determination is made with the bath maintained at 2-5 C below the cloud point obtained in the preliminary determination.

This paper describes some of the factors affecting such determinations. It provides an indication of the errors that could arise from conducting the test as described above. The repeatability and reproducibility standard deviations obtained through several collaborative trials also are reported.

METHOD

The reference method used in this study was the AOCS Method Cc 6-25.

MATERIALS

Samples studied include crude palm oleins, physically refined palm oleins, degummed bleached oleins and alkali refined oleins. The oleins were from the single fractionation process.

RESULTS AND DISCUSSION

Effects of Sample Container

The recommended container in the AOCS Method Cc 6-25 is vaguely defined as oil sample bottles, 115 ml, 4 oz. It is observed that different sample containers gave varying cloud point depending on the thickness of the walls or bottle diameter. Table I shows the difference obtained with different types of containers. Lower cloud point values were observed with thin walled vessels such as Nessler tubes, and higher values obtained with thicker walled vessels such as sample bottles. The repeatability of analysis varied depending on the thickness of the walls of the vessels. The greater fluctuations obtained with the use of beakers probably are due to some supercooling occurring in the thin walled vessel. Sample containers should therefore be strictly standardized.

Effect of Stirring Angle and Rate of Stirring

The effect of stirring at an angle as against upright stirring is

TABLE I

Effect of Container

	Cloud point °C	Standard deviation °C	No. of analyses
Sample bottle (2.5 mm thickness)	9.4	0.07	6
Beaker (2.0 mm thickness)	7.7	0.29	6
Nessler tube (1.8 mm thickness)	5.9	–	1