An Original Transesterification Route for Fatty Acid Ester Production from Vegetable Oils in a Solvent-Free System

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ABSTRACT: Wax ester production from a long-chain alcohol and methyl ester has been investigated with an immobilized thermostable lipase (lipozyme IM from Novo Nordisk). The transesterification reaction rate was monitored in solvent-free medium that was exclusively composed of the reactants and the enzyme. The transesterification is performed by simply mixing the two substrates in various stoichiometric amounts at a temperature range from 55 to 65°C under constant stirring in the presence of low concentrations of enzyme preparation (0.12 to 2%, w/w). Long-chain reactants produce waxes of high molecular mass that induce low solubility and high viscosity. On average, high transesterification yields are obtained (around 95%). Thermodynamic parameters involving substrate concentration and temperature have also been investigated. The balance between optimal working temperature and the molar ratio of substrates in such a complex medium appears to be 60°C, with a molar ratio methyl oleate/stearyl alcohol of 1:0.5. Substrate inhibition due to stearyl alcohol has been observed. A study of kinetic parameters has confirmed these results.
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KEY WORDS: Lipozyme, solvent-free system, transesterification, vegetable oils, wax esters.

Lipases catalyze many reactions of great interest, such as hydrolysis, synthesis, and inter- and transesterification (alcoholysis, acidolysis). These enzymes have been studied extensively in either aqueous or predominantly organic media. Changing the reaction medium, however, is a method of inducing reaction equilibrium shift. Because of the insoluble nature of many substrates in aqueous solution, enzymes can be usefully employed in organic solvents (1–3), multiphasic systems (4–6), micellar media (7), or with gas-phase substrates (8,9) and supercritical fluids (10). There has, however, been little research that deals with the direct utilization of enzymes on substrates (5,11–15). By this, we mean the biocatalyst acting directly on substrates with no extraneous solvents present. This method offers several advantages for improving substrate and product concentrations, selectivity of reactions and also for providing easier and fewer purification steps (15).

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From an economic standpoint, methyl esters from vegetable oils can be used as raw materials that require valorization. The use of long-chain substrates from vegetable oils allows the bioproduction of wax esters, which have many industrial applications in oleochemistry, such as lubricants and cosmetics (13). Compared to chemical methods, lipase-catalyzed reactions are better adapted to chemical ways. As a result of high enzyme selectivity and the presence of few side reactions, a reduction in undesirable by-products and lower overall reaction times have been obtained, compared to chemical transesterification. Biocatalysts also avoid molecular instability and diminish energy costs (temperatures up to 150–180°C are needed in chemical processes) (16,17).

The method of producing the wax ester (and methanol) is a transesterification reaction between methyl oleate (80% from methyl ester mix) and stearyl alcohol. The methanol evaporates at the working temperature (60°C) and hence its removal continually improves the reaction equilibrium and prevents the reverse reaction (18). Use of lipase has mainly been described in the context of hydrolytic and synthetic reactions. There are, to date, few reports in which lipases are used in transesterification reactions for wax production. Transesterification, described under such conditions, has been carried out for triolein production (6,11–13). Stearyl oleate has already been obtained by an esterification reaction between oleic acid and stearyl alcohol (6,13).

Many lipases have been tested for transesterification in low-water organic media. In a previous study (2), an alcoholysis reaction was catalyzed by three different lipases: porcine pancreatic lipase, *Candida cylindracea* lipase, and *Mucor* lipase. The catalytic activity of the *Mucor* lipase was independent of the percentage of water in the solvent. Additionally, much research has been carried out with a lipase from *M. miehei* in an immobilized form: lipozyme IM from Novo Nordisk. This enzyme has already been used in solvent-free reactions (11–13), and its utilization also provides other advantages. First, a minimal quantity of water is required to maintain the enzymatically active lipase conformation (19), and this immobilized form is supplied as a low-water content preparation. A study has reported the high retention of catalytic activity of this lipozyme, even at a water activity below 0.0001 (20). Second, its characteristic thermostability with regard to high medium viscosity and high melting tempera-

ture of reactants results in media constraints being imposed, and more often, studies are carried out at elevated temperatures. Third, the use of an immobilized enzyme reduces the number of purification steps required.

Our aim was to enzymatically produce waxes (long-chain esters) from stearyl alcohol and methyl oleate from vegetable oils. This work deals with investigating the effect of high substrate concentrations on the performance of the immobilized enzyme. The system is solvent-free. To assess the kinetic parameters, several variables have been investigated such as enzyme concentration, substrate effects, and temperature. Also, some thermodynamic properties have been evaluated to optimize operating conditions with regard to high concentrations of the long-chain reactants.

EXPERIMENTAL PROCEDURES

Chemicals. Stearyl alcohol, oleic acid methyl ester, and oleic acid stearyl ester were purchased from Sigma (St. Quentin Fallavier, France); *n*-heptane (99.9%) was obtained from Merck (Nogent-sur-Marne, France). Methyl esters of oleic sunflower (80% oleic acid methyl ester) and stearyl alcohol were supplied by Robbe (Compiegne, France). All others chemicals were purchased from Sigma. All reactions were catalyzed with a commercial lipase from *M. miehei*: lipozyme IM. This thermostable lipase is immobilized on a macroporous anion exchange resin and supplied with a low water content. The enzyme activity is expressed in Batch Acidolysis Units Novo per gram (BAUN/g). Its typical activity is 5–6 BAUN/g. The enzyme preparation was a generous gift from Novo Nordisk Bioindustrie S.A. (Nanterre, France).

Viscosity measurement. Viscosity measurements have been made for the initial substrate mix and the final product at three different controlled working temperatures. These measurements were carried out in a measuring cylinder with 50 mL reactant at 55, 60, and 65°C. The apparatus used is the Bohlin Visco 88 BV (Bohlin Instruments, Mühlacker, Germany).

Transesterification experiments. Stearyl alcohol had to be dissolved in the other substrate at a temperature not less than 55°C. Reactions for oleic acid stearyl ester synthesis from methyl esters of oleic sunflower or pure methyl oleate and stearyl alcohol have been performed by simply mixing the two reactants without any solvent in a temperature-controlled water bath under magnetic stirring in the presence of lipozyme from 0.12 to 2% w/w (percentage of the weight of the reaction mixture). Samples were taken at different intervals; the course of the reaction was followed by measuring the increase of product formed. The procedure was started by mixing the reactants (2 mL) at the required temperature for at least 15 min to ensure temperature equilibration, then lipozyme was added. Samples (10 μL) were removed (stirring stopped for 5 s), 10 μL internal standard was added to 1 mL heptane, and samples were subsequently analyzed by gas chromatography (GC).

GC analysis. The transesterification rate was periodically evaluated in a Hewlett Packard (Palo Alto, CA) Model 5890 series II GC, equipped with a flame-ionization detector (350°C) and a split injector (350°C, split ratio 60:1). The instrument was equipped with a 3396 Series II integrator. Two types of fused-silica capillary columns from SGE (Villeneuve St. Georges, France) were used. The first was an apolar HT5 column (12 m, 0.32-mm i.d., 0.1-μm film thickness), the second a moderately polar BPX35 column (12 m, 0.22-mm i.d., 0.25-μm film thickness). Nitrogen was used as a carrier gas with a flow rate of 30 mL/min. Column, hydrogen, and compressed air flow rates were, respectively, 3, 30, and 300 mL/min. Samples of solution (1 μL) were injected. The temperature program was: column temperature was set to 140°C for 1 min and then programmed to increase by 5°C/min to 350°C, which was maintained for 10 min. Product and reactant identification was achieved by comparing retention times to those of commercial standards; the amount of stearyl oleate was estimated by using an internal standard.

RESULTS AND DISCUSSION

This study describes the catalytic behavior of lipozyme IM in a solvent-free and low-water medium. The influence of initial catalyst concentration, substrate molar ratio, and reaction temperature on ester yield will be discussed with the aid of kinetic and thermodynamic parameters.

Before using methyl ester sunflower mix (the principal constituent being 80% methyl oleate, with 4% methyl palmitate and 10% methyl linoleate), the first study was concerned with pure commercial substrates, to determine the general working conditions. Figure 1 shows the synthesis of stearyl oleate and the consumption of stearyl alcohol and methyl oleate as a function of time (A) with pure substrates and (B) with methyl ester sunflower mix. These typical time-course experiments show that high conversion rates can be obtained. The substrates were a 1:1 molar ratio with 2% (w/w) enzyme at 60°C. In Figure 1A, with pure substrates, a yield of 93% was obtained after 210 min, and Figure 1B shows the total conversion of alcohol substrate in a mixture that was mainly composed of stearyl oleate (80%), stearyl palmitate (4%) and stearyl linoleate (10%), just like the initial methyl ester composition. A control experiment was performed (with no enzyme present for 24 h at 60°C) to ensure that no wax production had occurred. The uncatalyzed transesterification of methyl oleate into stearyl oleate under the same conditions accounted for only 0.4% of total conversion over the same period of time.

The effect of lipase concentration on wax production was tested. Generally, studies were performed with an enzymic preparation of 5 to 10% (w/w), and once with 2% (w/w) (22). For these experiments, reaction conditions were identical: only substrates in a 1:1 molar ratio were used with various concentrations of lipozyme from 0.12 to 2% (w/w) in open vials at 60°C. Two observations can be made from Figure 2. First, the transesterification yields are high, between 90 and 100% conversion in all runs, except when the lowest amount of enzyme was used. Second, increasing the amount of lipase,

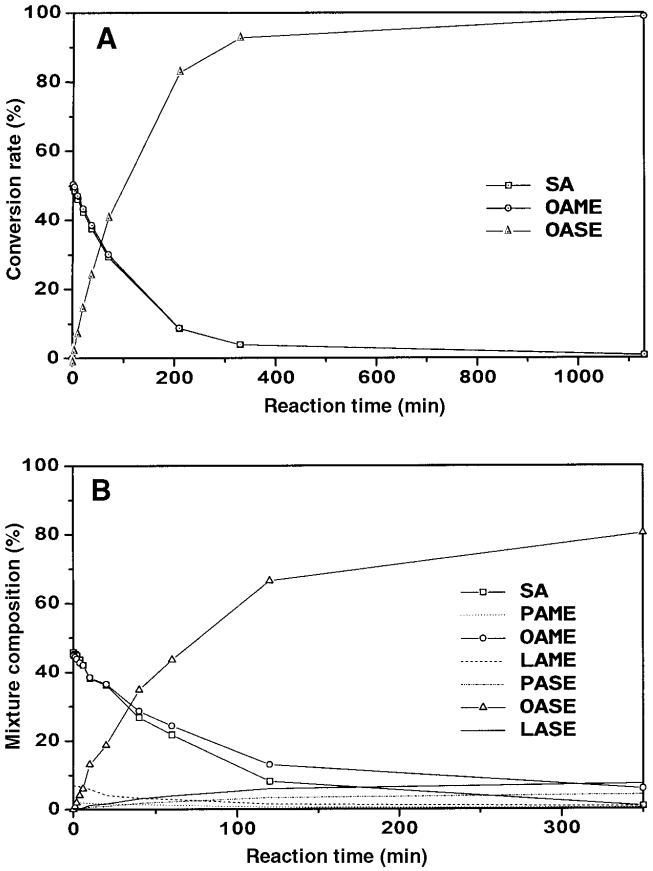


FIG. 1. Transesterification rate of (A) pure and (B) mixed substrates under stoichiometric conditions at 60°C with 2% (w/w) lipase. Abbreviations: SA, stearyl alcohol; OAME, oleic acid methyl ester; OASE, oleic acid steryl ester; PAME, palmitic acid methyl ester; LAME, linoleic acid methyl ester; PASE, palmitic acid stearyl ester; LASE, linoleic acid stearyl ester.

as expected, led to faster reaction rates for wax ester production. We observed that complete transesterification was reached when the initial lipozyme concentration was higher than 0.12% (w/w). Thus, the higher the effective enzyme concentration, the faster the reaction was, and with high enzymic preparation concentrations [2% (w/w)], high conversion rates could be obtained. The relationship between lipozyme concentration and initial reaction rate shows linear behavior (data not shown). This observed behavior apparently means that there was little or no diffusion limitation over the initial rate period. This linearity was obtained in spite of two facts: on the one hand, the medium consisted of raw materials (stearyl alcohol) in solid form at room temperature, which required elevated working temperatures (at least 55°C) with a concomitant decrease in medium viscosity, while on the other hand, an immobilized enzyme preparation was used.

We have studied the reaction rate as a function of alcohol substrate concentration (stearyl alcohol). As shown in Figure 3 for stearyl alcohol substrate concentrations of 26 and 432 mM, half-conversion rate was obtained in 10 min, and 75% alcohol conversion was reached within 30 min, except for the two highest concentrations of 1515 and 2130 mM. Thus, high substrate concentration appeared to slow product formation, probably owing to substrate inhibition.

We subsequently studied the effect of reactant ratio on the catalytic rate of the reaction. Several reactions with different substrate ratios were carried out to optimize experimental conditions. The melting point of stearyl alcohol and oleic acid methyl ester are, respectively, ~60 and −19.9°C. Thus, when the ratio of stearyl alcohol/oleic acid methyl ester is increased, viscosity should be borne in mind. The working temperature of 55°C was chosen because of two considerations. First, this elevated temperature allowed us to avoid the problem of stearyl alcohol solubility in oleic acid methyl ester and also reduced the viscosity, and second, the enzyme activity at this elevated temperature was still unaffected. However, the process of immobilizing enzymes is known to increase lipase thermostability (14). Lipozyme IM can be used over a temperature range from 30 to 70°C without loss of activity, but the lower the reaction temperature, the greater the expected enzyme stability. The influence of stearyl alcohol concentration on the initial rate of stearyl oleate formation at different temperatures was studied. In Figure 4, when the initial alco-

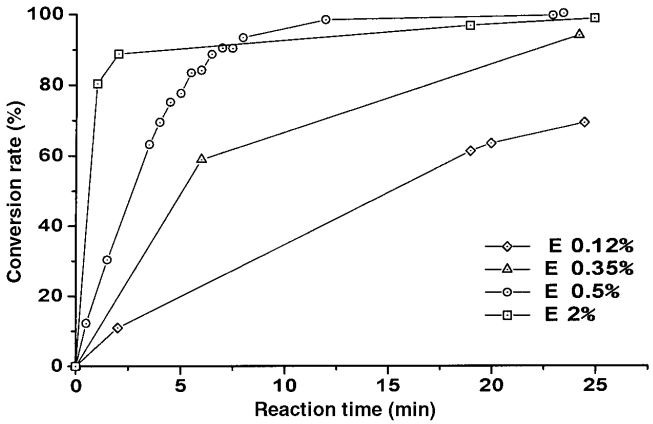


FIG. 2. Transesterification yield as a function of reaction time and quantity (E) of lipase [0.12 to 2% (w/w), percentage of the weight of the reaction mixture, under stoichiometric conditions at 60°C].

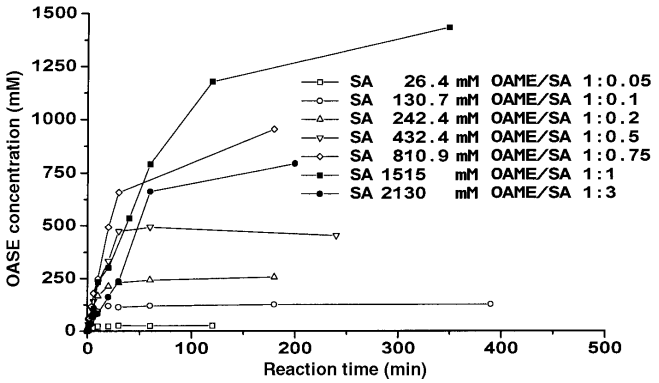


FIG. 3. OASE production as function of reaction time and substrate concentration of SA at 60°C with 2% (w/w) lipase. For abbreviations see Figure 1.

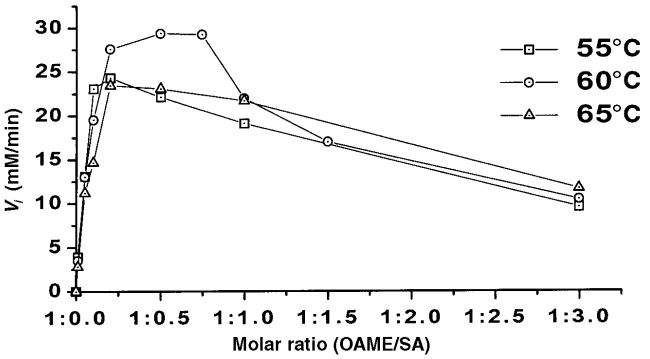


FIG. 4. Initial rate of stearyl oleate production as a function of different ratios of OAME/SA, at 55, 60, and 65°C. V_i is the initial rate of stearyl oleate formation. For abbreviation see Figure 1.

hol molar ratio is increased above 1:0.2 for 55 and 65°C and 1:0.5 for 60°C, a decline in the initial reaction rate is observed, which indicates alcoholic substrate inhibition. However, the decrease in the efficiency of transesterification at high alcohol concentrations may also be due to mass transfer problems. This decrease restricts the range of substrate concentrations that can be used in determining apparent K_M (affinity constant) and V_M (maximal rate) values. The best initial velocity is obtained at 60°C with a methyl oleate/stearyl alcohol molar ratio of 1:0.5. At 60°C in the open vial, methanol is evaporated during the transesterification reaction, and methyl oleate may act as a co-solvent of alcohol.

Apparent kinetic parameters have been estimated, and two facts have to be underlined. First, this kinetic approach is performed with the initial velocity, to ensure that no other compound from the mixed substrates reacts with alcohol. Second, under the working conditions (only substrates without solvent), the concentration of one substrate depends on the concentration of the other substrate (nonindependent variable), and both are bound. In Figure 5, we have plotted the curves (V_i, methyl oleate) = f (stearyl alcohol). Increasing stearyl alcohol concentration led to a decline in velocity. This model is close to a standard model. Kinetic parameters have been estimated from Lineweaver-Burk plots. To assess the probability of substrate inhibition, we have plotted in Figure 6 the curves $1/V_i$ vs. 1/[methyl oleate] and $1/V_i$ vs. 1/[stearyl alcohol]. In Figure 6A, the plot is linear for stearyl alcohol concentrations

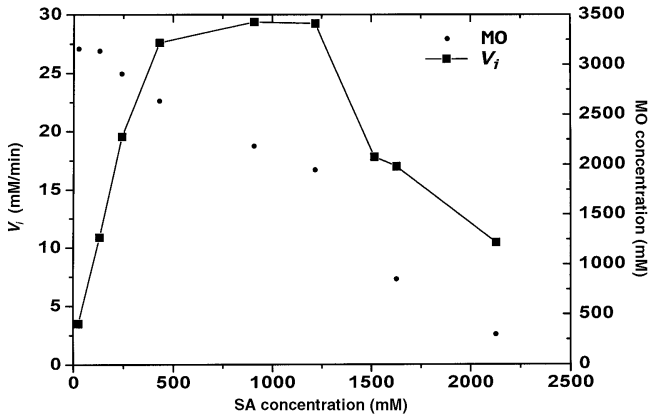


FIG. 5. Initial rate of stearyl oleate production and methyl oleate (MO) concentration as a function of SA concentration at 60°C, with 2% (w/w) lipase. See Figure 1 for abbreviation.

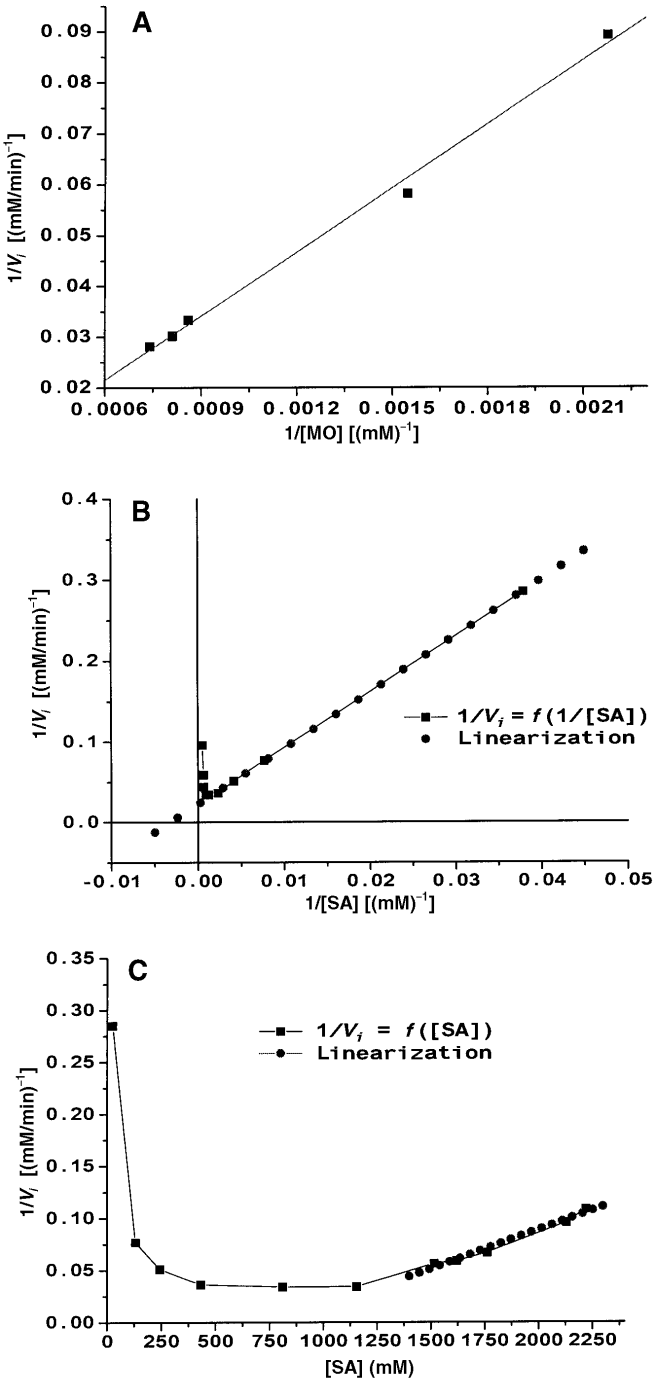
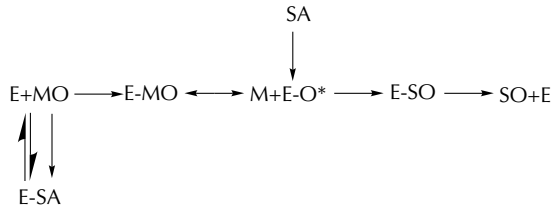


FIG. 6. Representation of Lineweaver and Burk equation, $1/V_i = f(1/[\text{substrate concentration}])$, at 60°C with 2% (w/w) lipozyme. (A) Kinetic plot $1/V_i = f(1/[MO])$, for different MO concentrations; (B) Kinetic plot $1/V_i = f(1/[SA])$, low values; SA concentrations are varied; (C) Kinetic plot $1/V_i = f(1/[SA])$, high values; SA is varied. See Figures 1 and 5 for abbreviations.



E = lipase; MO = methyl oleate; M = methanol; SA = stearyl alcohol; SO = stearyl oleate

SCHEME 1

from 459 to 1400 mM. In Figure 6B, alcohol substrate inhibition is confirmed. These curves can be characteristic of a stearyl alcohol substrate inhibition in a ping-pong ordered bi-bi system (25). One product, the methanol, is released between the addition of the two substrates. The study domain, in terms of concentration, is limited, which does not lead to a resolution of competitive or uncompetitive alcohol inhibition. The reaction sequence (25) is represented in Scheme 1.

The velocity equation is:

$$V_m/V = 1 + K_M\cdot sa/[SA] + (1 + [SA]/K_i) \cdot K_M\cdot mo/[MO] \quad [1]$$

When the concentration of methyl oleate is varied, the equation can be written (Fig. 6A):

$$1/V_i = ([MO] + K_M\cdot mo.app)/V_M\cdot app\ [MO] \quad [2]$$

where $V_M\cdot app = V_M/(1 + K_M\cdot as/[SA])$ and $K_M\cdot mo\cdot app = \{K_M\cdot mo\ (1 + [SA]/K_I)\}/(1 + K_M\cdot sa/[SA])$. When the concentration in stearyl oleate is varied, the velocity equation is given by:

$$1/V_i = [SA]\ \{(1 + (K_M\cdot mo/[MO]) \cdot (1 + [SA]/K_I)\}/(V_M \cdot [SA]) \quad [3]$$

Two situations have appeared, depending on the stearyl alcohol concentration: (i) For low stearyl alcohol concentrations, the velocity equation is (Fig. 6B):

$$1/V_i = (1 + K_M\cdot sa\cdot app)/(V_M\cdot app \cdot [SA]) \quad [4]$$

with $K_M\cdot sa\cdot app = K_M\cdot sa \cdot \{[MO]/([MO] + K_M\cdot sa)\}$ and $V_M\cdot app = V_M/(1 + K_M\cdot mo/[MO])$. (ii) For high stearyl alcohol concentrations, the velocity equation is given by (Fig. 6C):

$$1/V_i = (K_I\cdot app + [SA])/(K_I\cdot app \cdot V_M\cdot app) \quad [5]$$

with $K_I\cdot app = K_I \cdot (1 + K_M\cdot mo/[MO])$ and $V_M\cdot app = V_M \cdot (1 + K_M\cdot mo/[MO])$.

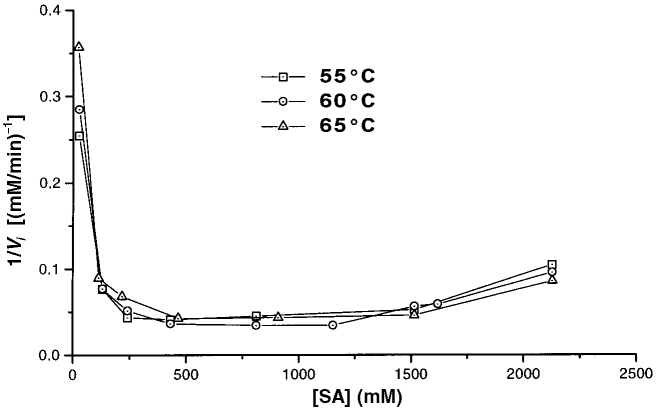


FIG. 7. Reciprocal initial rate vs. SA concentration at 55, 60, and 65°C [2% (w/w) lipase]. See Figure 1 for abbreviation.

In any case, linearization of plots gives apparent kinetic data. These parameters are presented in Table 1. The orders of magnitude of $K_M\cdot mo\cdot app$ and $V_M\cdot mo\cdot app$ seem of no value and are too high. At the first reaction step, stearyl alcohol does not interfere except for the inhibition step, binding stearyl alcohol with lipase instead of methyl oleate. Moreover, these apparent affinity constants are related to K_I and $K_M\cdot sa$.

To assess the probability of substrate inhibition, we have plotted in Figure 7 the curve $1/V_i = f$ (stearyl alcohol) at three different temperatures. The data confirm that substrate inhibition occurs and also give complementary information, such as K_I (substrate inhibition constant). These values are summarized in Table 1. K_M is a constant for a given enzyme under determined conditions. Because the catalytic efficiency of the system is optimal for the highest V_M/K_M value (23), the kinetic parameters suggest that 60°C is the optimal temperature, with a good balance between physicochemical complexity of the medium (viscosity problems), lipozyme IM activity at elevated temperature, and evaporation of the methanol product formed. These results confirm those shown in Figure 4 in which V_i was plotted as a function of molar ratio (varied concentrations of alcohol) at 60°C, where not only are the V_i values the highest but also the apparent V_M values. Regarding the K_I constants, at 55, 60, and 65°C, the three are within the same magnitude: 899 to 800 mM. We observe that the lower the temperature, the higher is the $K_I\cdot app$. Thus, increasing the working temperature does not lead to a decrease in substrate inhibition. Moreover, the apparent value of K_M di-

TABLE 1
Apparent Kinetic Constants[a]

Temperature (°C)	$K_M\cdot mo\cdot app$ (mM)	$V_M\cdot mo\cdot app$ (mM/min)	$K_M\cdot sa\cdot app$ (mM)	$V_M\cdot sa\cdot app$ (mM/min)	$V_M\cdot app/K_M\cdot app$ (min^{-1})	$K_I\cdot app$ (mM)
55	—	—	322	44.7	0.138	899
60	11 447	280	315	45.4	0.144	871
65	—	—	258	33.0	0.128	800

[a] $V_M\cdot app$: apparent maximal rate; $K_M\cdot mo\cdot app$: apparent affinity constant for methyl oleate; $K_M\cdot sa\cdot app$: apparent affinity constant for stearyl alcohol; $K_I\cdot app$: apparent affinity constant of inhibition.

minishes with temperature, suggesting that the apparent affinity is augmented with temperature. This would be an indicator of a diffusion effect. A first approach, due to physicochemical complexity of the medium, has been performed by measuring solution viscosity (data not shown). These measurements have been made for the three working temperatures, both for the initial substrate mix and for the final products. On the whole, the values of viscosity are low and close to the detection limits of the apparatus. Thus, at working temperatures, viscosity does not limit the transesterification reaction. Another study confirms that at 60°C the maximum initial rate was obtained with esterification between oleic acid and glycerol as substrates (24). Although advantages have been offered for using enzymes in solvent-free systems, the application can appear to be limited in regard to high substrate melting points and adequate enzyme stability at higher temperatures as confirmed in classical enzymology. However, it has been demonstrated recently that the use of enzymes under conditions of low water content allows high enzyme activity and also high enzyme stability because water is an intrinsic part of the denaturation process. In a trial run, reusing the enzyme after washing it with solvent at 60°C gives the same rate as a function of time. This observation again suggests high enzyme stability at 60°C under our conditions.

Thermodynamic analysis has been employed to explain physicochemical observed effects. The effect of temperature on enzyme activity can be twofold: reduction of the activation energy by increasing the temperature (Arrhenius' law), and denaturation of the protein structure by heat. An assessment of the activation energy may be obtained from the initial reaction rate as function of temperature. The lipase acts by reducing the activation energy. In the Arrhenius plots of log V_m vs. reciprocal temperature, we observed, compared to the classical linear dependence, a break that corresponds to the critical temperature within the range of 55–65°C. We attribute this to the physicochemical complexity of the medium.

This paper has demonstrated that high transesterification rates can be obtained, even with low concentrations of enzymatic preparations, by using pure substrates in the absence of added solvent. Optimization of molar ratio and temperature has been investigated. The balance between these two parameters in such a medium gives the optimum working conditions as 60°C with a molar ratio of oleic acid methyl ester/stearyl alcohol of 1:0.5. It has been possible to obtain with our system high conversion rates that approach 100% in 30 min at 60°C. The enzyme can be reused with the same transformation yield. In chemical synthesis reactions, conversions of 50–70% can be obtained after longer reaction times (6 h) at 150–180°C, and a significant yield of by-products appears during the reaction (17). Our procedure is simple, and the use of medium with low water activity allows a good working system in the transesterification reactions and is associated with good enzyme stability.

We used no organic solvents. The oleic acid stearyl ester can be qualified as a "green product" and has the advantage of a natural label. This wax ester can be used without difficulty in the cosmetic, pharmaceutical, agro-food, and fine chemistals industries.

We are currently developing a system that can be used as a continous-feed reactor. Such a system may permit eliminating substrate inhibition. Moreover, the solid/liquid reaction medium, in which the supported enzyme constitutes the solid form and substrates and products used at temperatures higher than 55°C constitute the liquid form, are perfectly adapted to the construction of an open column-type reactor.

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