Enzymatic Synthesis of Wax Esters from Rapeseed Fatty Acid Methyl Esters and a Fatty Alcohol

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ABSTRACT: Wax esters were transesterified from fatty acid methyl esters of rapeseed and a fatty alcohol (1-hexadecanol, 16:0). The amounts of both the substrates were fixed to 0.1 mmol and an immobilized enzyme, Lipozyme, was used as catalyst. The experiment was performed following a statistic central composite design with five variables. The enzyme/lipid ratio was varied between 0.3–0.9 of the substrate weight and the enzyme was equilibrated to different water activities varying from 0.11 to 0.44. A temperature range of 50–80°C was investigated and the reaction time lasted up to 40 min. A solvent, isooctane, constituted 0–30% of the substrate weight. The first experimental series was performed in small closed test tubes. In the second series the caps of the test tubes were off to evaporate the methanol produced during the reaction. The highest initial reaction rate was 9.6 $g_{wax\ esters}/g_{enzyme} \cdot h$. It appeared when: the enzyme/lipid ratio was low, 0.3, the temperature was high, 80°C; no isooctane was present; and the water activity was below 0.11. The initial reaction rate was independent of the caps on the test tubes. With the large amount of enzyme the yield of wax esters was above 70% after 10 min in both experimental series. In the reaction with caps, the reaction reached equilibrium at 83% after 20 min at 80°C. However, without caps the continuous evaporation of methanol increased the equilibrium constantly, and after 40 min at 80°C a yield of 90% was reached.

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KEY WORDS: Fatty acid methyl esters, Lipozyme, methanol evaporation, rapeseed oil, statistic experimental design, wax esters.

Commercial waxes have a wide range of uses as lubricants, polishes, plasticizers, coating materials in the medical and food industries, and as raw materials in cosmetic and other chemical industries (1). The natural waxes originate from animals, vegetables, and minerals. Many of the important commercial waxes contain rather high percentages of saturated wax esters (WE), such as beeswax. WE are esters of long-chain fatty acids and long-chain fatty alcohols. Traditional raw material for unsaturated WE are sperm whale and jojoba oil. Since these waxes are expensive and limited in access, the need to synthesize unsaturated WE has grown.

There are different ways of synthesizing WE. The two most common are chemical synthesis (2,3) and enzymatic synthesis (4–10). Enzymatic synthesis uses lower temperatures than chemical synthesis and a single product is produced at a higher yield. There are three main reactions which can be used: esterification, transesterification, and alcoholysis. The reactions are performed in organic solvents, and the water content has to be controlled at a very low level (11) or the enzyme recovers its hydrolytic activity (12).

Fatty acid methyl esters (FAME) have only been used as starting materials for synthesis of WE in a very few reports. Charlemagne and Legoy (9) reported an enzymatic synthesis of polyglycerol-fatty acid esters in a solvent-free system *via* transesterification. They used FAME from a vegetable oil as starting materials to react with polyglycerol to produce esters. Evaporation of methanol formed during the reaction increased the yield, and the use of silica for adsorption of the polyglycerol prior to transesterification improved the yield further. For all the methods there is always a risk of losing enzyme activity by removing water, methanol or ethanol, and glycerol formed during the reaction. However, it would be easier to remove methanol or ethanol from the reaction system to improve the yield, instead of removing water.

We have produced WE by enzymatic synthesis using FAME from rapeseed and a fatty alcohol (1-hexadecanol, FOH) (see Equation 1).

$$\text{FAME} + \text{FOH} \rightarrow \text{WE} + \text{MeOH} \qquad [1]$$

The reaction was catalyzed by immobilized lipozyme in an organic media (isooctane). We have used a fractional factorial experimental design to describe the initial reaction rate (IRR) and the yield of WE with and without evaporating of the methanol formed during the reaction. Five variables were examined: the temperature, the time, the amounts of isooctane and enzyme, and the water activity (a_w) of the enzyme.

MATERIALS AND METHODS

Materials. Rapeseed FAME were obtained from Larodan (Malmö, Sweden) and cetyl alcohol (1-hexadecanol, FOH) was bought from Sigma Chemical Co. (St. Louis, MO). Im-

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mobilized enzyme from *Mucor miehei*, Lipozyme® IM (triglyceride hydrolase, EC 3.1.1.3), was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). The isooctane was of analytical grade and the chemicals for analysis by high-performance liquid chromatography (HPLC) were of HPLC grade. They were all bought from Merck (Darmstadt, Germany).

Experimental design and modeling. The experiments were performed using a statistical experimental design. The levels of the variables (temperature, time, isooctane, enzyme, and a_w of the enzyme) were selected according to a central composite design with fractional factorial design ($2^{5-1} + 2 \times 5 + 2 = 28$ exp.) (13). The values of the five variables are shown in Table 1. The isooctane and the enzyme are presented as weight percentage of the total substrate. The experiments were run in two series. In the first series the reactions were performed in small closed test tubes. In the second series the caps on the test tubes were off. The amounts of the substrates FAME and FOH were kept constant at 0.1 mmol each (a total of 51.6 mg).

Equilibration of the immobilized enzyme at different water levels. The immobilized enzyme was dried over silica (Blue gel). The water concentration of the immobilized enzyme was controlled by equilibration over four saturated salt solutions with defined a_w. The salt solutions were kept in desiccators under vacuum at room temperature. The dried immobilized enzyme was equilibrated over the salt solutions for at least a week before the first sample was taken. The salts chosen and their a_w (11,14,15) are shown in Table 2. The a_w of Blue gel, dried to 105°C, was found to be 0.06 by interpolation of the water content between $a_w = 0.11$ (LiCl) and P_2O_5, where P_2O_5 is set to $a_w = 0$.

Samples were taken from the enzyme of each a_w, and the water content was analyzed before the experimental series were started and after they were completed, by drying samples for 2 h at 110°C. The mean values are given in Table 2. The water content of the fractions changed less than ±10% during the experiment. The uncertainty in the figures was more related to the analysis procedure than to a real variation in the water content.

Preparation of the reaction mixture. FAME (27.4 mg) and FOH (24.2 mg) (= 0.1 mmol each) were weighed into a small test tube with a screw cap. In the second experimental series the screw cap was left off. In each experiment the amounts of isooctane and immobilized enzyme of the right a_w were added according to the experimental design. The tubes were put in a shaker at the desired temperature for various periods of time. At final time, 1 mL of isooctane was added to the sample. The tube was shaken and the sample was withdrawn without the enzyme into an HPLC vial.

TABLE 1
Experimental Design

Variables	Levels				
	Very low	Low	Medium	High	Very high
Temperature (°C)	32	50	65	80	98
Time (min)	4.3	10	20	40	93
Isooctane (%, w/w)	0	0	15	30	48.5
Enzyme/lipid ratio[a]	0.07	0.3	0.6	0.9	1.27
Water activity of the enzyme	0.06	0.11	0.33	0.44	0.75

[a]mg enzyme/mg substrate.

TABLE 2
Saturated Salt Solutions, Their Water Activity and the Water Content of the Immobilized Enzyme After Equilibration Above the Saturated Salt Solutions for a Week Under Vacuum (Ref. 14)

Saturated salt solution	Water activity (at 20°C)	Water content of the enzyme
Silica (Blue gel)	0.06	1.2%
Lithium chloride (LiCl)	0.11	5.2%
Magnesium chloride ($MgCl_2$)	0.33	9.4%
Potassium carbonate (K_2CO_3)	0.44	12.0%
Sodium chloride (NaCl)	0.75	16.0%

HPLC analysis. The composition of the reaction mixture in terms of WE, FAME, and FOH were analyzed by HPLC on a Shimadzu SCL6B equipped with a diol column (Lichrospher 100 diol, 5 µm, 4 × 250 mm, Merck) and a Light Scattering Mass Detector 750/14 (ACS). The sample was eluted using a gradient of heptane, isopropanol, and acetic acid as described before (16).

Evaluation. The experimental data were evaluated by models including all linear, quadratic, and cross-correlating terms up to the second degree for the independent variables, and the yield was the dependent variable. The estimation and the graphical presentation of the models were made using the computer program SYSTAT (17). The precision of the models was given by standard error of estimate (SEE), which includes the experimental error and the error of the model. All of the experimental data were found to be within the range k*SEE. A deviation had to be larger than k*SEE to be considered significant. In the plots, we restricted the evaluation range from the low level to the high level for every variable (see Table 1). If very high or very low levels were included in the plots, the uncertainties increased.

The yield of WE was calculated as the molar ratio of the formed amount of WE to the initial amount of FOH, i.e., in Equation 2 WE and FOH stand for the analyzed molar amounts in the samples from the reaction mixture,

$$\text{yield of WE} = 100 \times \text{WE}/(\text{WE} + \text{FOH}) \qquad [2]$$

The initial reaction rate (IRR) was calculated from the estimated yield at 10 min and expressed as produced WE per amount of enzyme and time ($g_{\text{wax esters}}/g_{\text{enzyme}} \cdot h$).

RESULTS AND DISCUSSION

This work was performed with a statistical experimental design. Several experiments were made to choose the values of the variables: temperature, time, isooctane, enzyme, and initial a_w of the enzyme, shown in Table 1.

During the reaction, methanol was formed (see Eq. 1). Preliminary results showed that MeOH interfered with the trans-

esterification reaction. This was also reported by Charlemagne and Legoy (9). To investigate if the yield of WE increased when the formed MeOH was evaporated, the experiment was run in two series, one with the screw caps on the test tubes and one without the caps.

IRR. IRR was described as a function of temperature, enzyme amount, initial a_w, and isooctane concentration. Plots of the IRR for this reaction are shown in Figures 1A and 1B. In Figure 1A the temperature and the enzyme/lipid ratio were varied while the initial a_w and the isooctane concentration were kept at their most favorable values (0.11 and 0%). In both series the highest IRR was 9.6 $g_{wax\ esters}/g_{enzyme} \cdot h$. It was found when the temperature was high (80°C), and the levels of enzyme, a_w and isooctane concentration were low (enzyme/lipid ratio = 0.3, a_w = 0.11 and isooctane concentration=0%) as can be seen in Figures 1A and 1B. At a low enzyme concentration, the contact between the enzyme and the substrate is better; thus the IRR increases when the enzyme ratio decreases. Without enzyme there was no reaction at all. The IRR also increased when the temperature was raised from 50 to 80°C (see Fig. 1A).

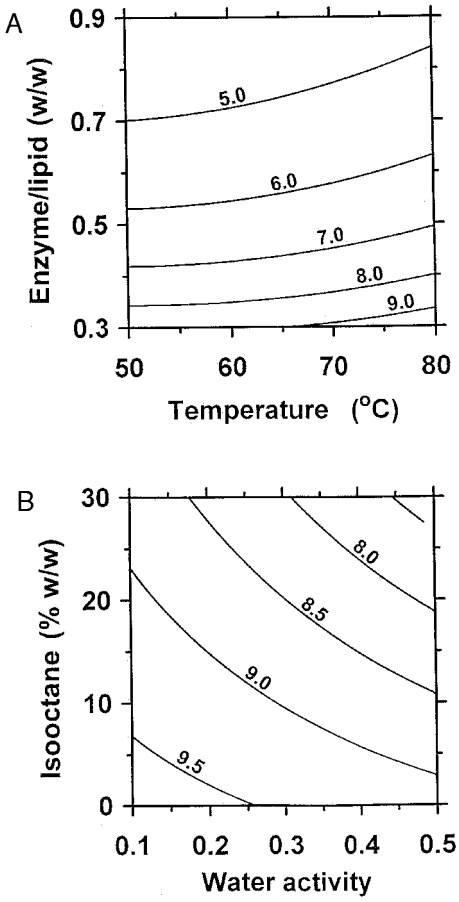


FIG. 1. (A) The initial reaction rate (IRR; $g_{wax\ esters}/g_{enzyme}h$) in the reaction with the caps on at different temperatures and enzyme/lipid ratios, when the other variables were held constant: water activity = 0.11 and isooctane concentration = 0% (w/w) (1.2 × SEE = 0.07). (B) The IRR in the reaction with the caps on at different water activities and isooctane concentrations, when the other variables were held constant: enzyme/lipid ratio = 0.3 (mg Enz/mg Sub) and temperature = 80°C (1.2 × SEE = 0.07).

In Figure 1B the initial a_w and the isooctane concentration were varied instead, while the temperature was kept steady at 80°C and the enzyme/lipid ratio was kept at 0.3, which was found to be the best value for the IRR (see Fig. 1A). The IRR increased when the isooctane concentration was lowered from 30 to 0%, as can be seen in Figure 1B, and the IRR increased when the initial a_w decreased, from 0.5 to 0.1 (see Fig. 1B). This is not in agreement with Valivety *et al*. (12), who reported a maximum reaction rate of around a_w of 0.5 for the enzyme lipozyme when decanoic acid was esterified with dodecanol in a higher amount of organic solvent than we used.

No difference was found on the IRR between the two experimental series, with and without caps.

Fatty acids. Besides WE and methanol, some small amounts of fatty acids were formed. The formation of fatty acids during the reaction with the caps on is shown in Figure 2.

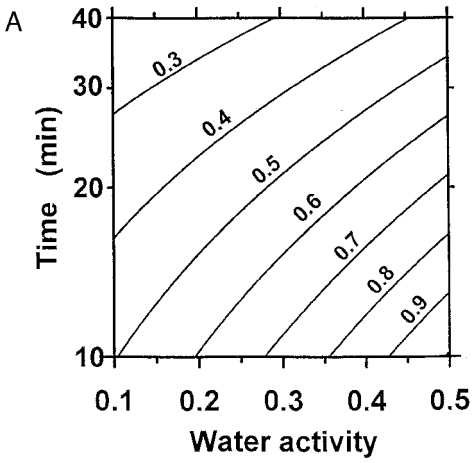
The amount of fatty acids was below 1% in all cases. However, the reaction rate was high and the maximal level was reached in less than 10 min. When the a_w was raised, the concentration of fatty acids increased strongly (see Fig. 2A). A temperature rise from 50 to 80°C also increased the concentration of fatty acids (see Fig. 2B). When the wax esters synthesis reaction proceeded and the FAME concentration decreased, the fatty acid concentration decreased (see Fig. 2A, the time scale, and Fig. 2B, the enzyme scale).

Thus, the enzyme becomes saturated with fatty acids from FAME. Water molecules release the fatty acids from the enzymes in a few minutes. At this stage, a_w determines the concentration level of the fatty acids. Later, when the fatty acids in the FAME have been converted to WE, the fatty acid concentration is lower at the same a_w. Thus, the enzyme is not saturated with fatty acids any more. The turnover rate in Equation 3 is much higher than in Equation 4. These statements were also supported by the results from the series without caps.

$$\text{FAME} + H_2O \leftrightarrow \text{FA} + \text{MeOH} \quad [3]$$

$$\text{WE} + H_2O \leftrightarrow \text{FA} + \text{FOH} \quad [4]$$

Yield of WE. Figure 3 illustrates the results from the series with the caps on and Figure 4 illustrates the yield of WE from the series without caps. In Figures 3A and 4A the temperature and the time were varied. The initial a_w was kept at the low value, 0.11, where the formation of fatty acids was lowest. The isooctane concentration and the enzyme/lipid ratio was kept at the high levels, 30% and 0.9, to reach the equilibrium of WE in a short time. With this large amount of enzyme the yield was above 70% after 10 min in both experimental series (see Figs. 3A and 4A). In the reaction with the caps on, the reaction rate had reached equilibrium after 20 min; after 20 min there was no significant change in the yield (see Fig. 3A). The equilibrium level was 75% at 50°C and 83% at 80°C. However, the yield of WE was overall 5–10% higher



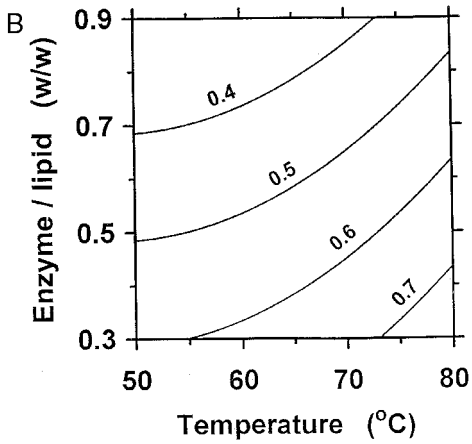
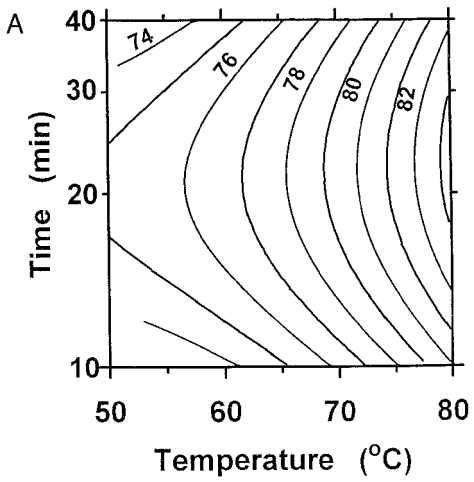


FIG. 2. (A) The fatty acid concentration (%mol/mol) in the reaction with the caps on at different reaction times and water activities. The following variables were held constant: isooctane concentration = 0% (w/w), enzyme/lipid ratio = 0.3 (mg·Enz/mg·Sub), and temperature = 80°C (1.3 × SEE = 0.12). (B) The fatty acid concentration (%mol/mol) in the reaction with the caps on at different temperatures and enzyme/lipid ratios. The following variables were held constant: isooctane concentration = 0% (w/w), water activity = 0.33, and time = 10 min (1.3 × SEE = 0.12).



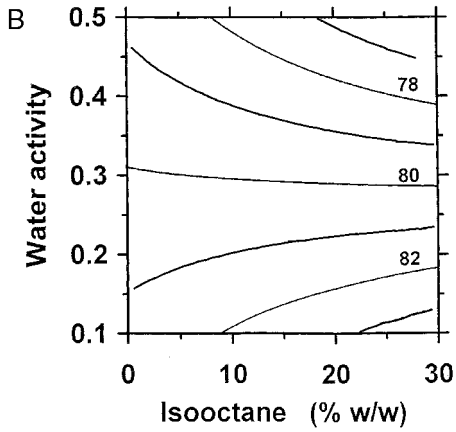


FIG. 3. (A) Yields of wax ester (%mol/mol) in the reaction with the caps on at different temperatures and times. The following variables were held constant: isooctane concentration = 30% (w/w), water activity = 0.11, and enzyme/lipid ratio = 0.9 (1.2 × SEE = 0.37). (B) Yields of wax ester (%mol/mol) in the reaction with the caps on at different isooctane concentrations and water activities. The following variables were held constant: temperature = 80°C, time = 22 min, and enzyme/lipid ratio = 0.9 (1.2 × SEE = 0.37).

in the experiment without caps (see Fig. 4). After 20 min the yield was already 83% at the low temperature of 50°C, and it reached 90% after 40 min at the high temperature of 80°C, as can be seen in Figure 4A. The continuous evaporation of formed methanol increased the equilibrium yield constantly.

a_w *and isooctane*. Since the substrate mixture melted at 53°C, the isooctane was needed to dissolve the substrates at the lower temperatures but not at higher temperatures. In Figures 3B and 4B the yield of WE is shown when the isooctane concentration and the initial a_w were varied. The temperature and the enzyme/lipid ratio were kept at their high levels (80°C and 0.9, respectively).

The figures were drawn close to the equilibrium times for the reactions, i.e., at 22 min for the series with caps on (Fig. 3B) and at 40 min for the series without caps (Fig. 4B).

In the reaction with caps on (Fig. 3B) the yield of WE increased when the initial a_w decreased, both at high and low concentrations of isooctane. The highest yield was found when the isooctane concentration was at the high level, 30%, and when the inital a_w was low, 0.1. In the reaction without caps the highest yield was found at the same conditions. However, at low concentrations of isooctane, below 15%, the initial a_w had no influence on the yield of WE (Fig. 4B).

It was easier to keep the a_w constant in the closed test tubes. Maybe water evaporated with the methanol when the caps were off and no isooctane was present. Probably, an initial a_w lower than 0.11 was the right range for the enzyme in this reaction with isooctane used as solvent.

Both Figures 3B and 4B show that the presence of isooctane increased the yield at high amounts of enzyme, i.e., a solvent increased the maximal yield. However, the IRR was favored with low enzyme concentration and no isooctane (see Fig. 1B). Thus, a solvent was needed to increase the contact between enzyme and substrate when the concentration of product was high.

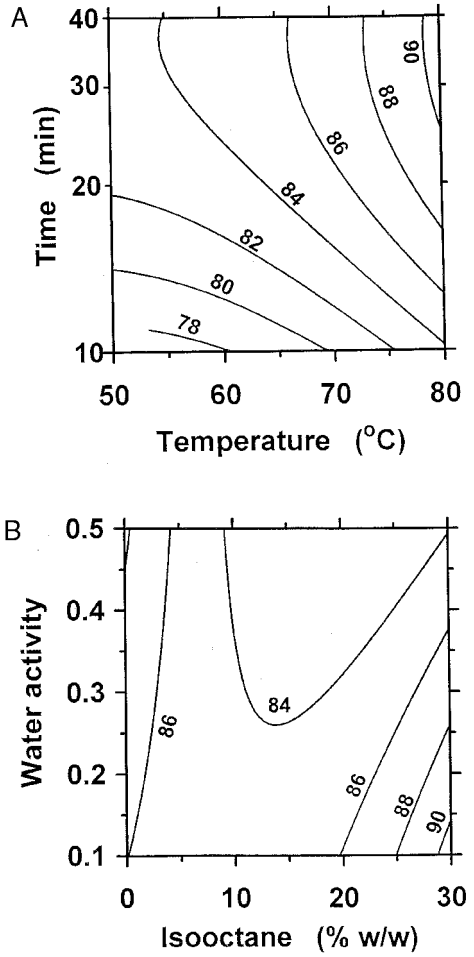


FIG. 4. (A) Yields of wax ester (%mol/mol) in the reaction without caps at different temperatures and times. The following variables were held constant: isooctane concentration = 30% (w/w), water activity = 0.11, and enzyme/lipid ratio = 0.9 (1.1 × SEE = 2.2). (B) Yields of wax ester (%mol/mol) in the reaction without caps at different isooctane concentrations and water activities. The following variables were held constant: temperature = 80°C, time = 40 min, and enzyme/lipid ratio = 0.9 (1.1 × SEE = 2.2).

Our experiment shows that WE could be easily produced by enzymatic synthesis from FAME and fatty alcohols using an immobilized enzyme as catalyst. The best results are achieved at an initial a_w of about 0.11 and at a temperature of about 80°C. The enzyme is most efficient at high substrate concentration, but a high WE concentration restricts the efficiency. The equilibrium is reached in a shorter time if a solvent is present. If the methanol formed is evaporated, the equilibrium and the maximal yield will continuously be increased from 83 to above 90%. In future experiments we will further evaporate the methanol and optimize the conditions to increase the reaction rate and determine the inactivation rate for the enzyme.

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