Reactive Extraction of Acylglycerides Using *Aspergillus flavus* Resting Cells

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ABSTRACT: A one-pot TAG extraction and FAME formation using fungal resting cells and oilseeds at moderate temperature are described. The final yield of methyl esters is increased by the sequential addition of water and methanol. The process can be carried out either with solvents or in a solvent-free system. When a solvent-free medium is used, the final yield will increase if soft oilseeds are used.

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KEY WORDS: *Aspergillus flavus*, biodiesel, bound lipase, fat extraction, fatty acid, fungal resting cells, lipase, methyl ester, reactive extraction.

FA and some of their derivatives, such as soaps and fatty esters, have multiple industrial applications. These include the preparation of food additives, functional foods, personal-care products, drugs, oil additives (1), and, more recently, biodiesel (2).

Both fatty acids and derivatives can be prepared from acylglycerides using either chemical or biocatalytical methods (3). Although acylglycerides are of plant or animal origin, they can also be obtained from waste materials (e.g., from vegetable oil industries and frying oils).

Acylglycerides are usually extracted from plant or animal material using physicochemical or physical methods. Crude extracts are then purified and finally modified (4). The interest in using biocatalysts to carry out these modifications is rapidly increasing and supported by people concerned about conventional chemical methods (5).

Among biocatalysts, lipases are the enzymes most frequently used to carry out these transformations. Although most of the reports have been on extracellular lipases, resting cells displaying lipase-like activity also have been used (6). Fungal resting cells are economically attractive since they are inexpensive to produce. The recovered biomass can be used directly as biocatalyst, avoiding isolation, purification, or immobilization procedures (7).

The use of fungal resting cells to transform TAG already has been proposed (8). However, no one has previously proposed taking advantage of the mild conditions associated with these transformations. They should permit one-pot extraction and transformation of acylglycerides to the desired compounds without modifying the properties of nonlipidic material present in the seeds. Rüsch gen. Klass and Warwel (9) recently proposed using a commercial immobilized enzyme (Novozym 435), deposited at the bottom of a Soxhlet apparatus, to transesterify TAG extracted from the oilseed-reservoir using either dimethyl or diethyl carbonate. Rüsch gen. Klass and Warwel call this process a reactive extraction.

A reactive extraction method has been developed in our attempt to develop environmentally friendly methods using fungal resting cells as a biocatalyst. The method permits one-pot extraction and transesterification of the acylglycerides present in various oilseeds. Moreover, the fungal resting cells, which are much cheaper than immobilized enzymes, and the defatted plant material can be a subsequent source of feed protein.

EXPERIMENTAL PROCEDURES

Chemicals. Trifluoroacetic acid (99.5%) was purchased from Fluka (Alcobendas, Spain). Isooctane and hexane were obtained from Panreac (Barcelona, Spain). Methanol was purchased from Scharlab (Barcelona, Spain). HPLC-grade acetonitrile and acetone were from Baker (Deventer, The Netherlands). All chemicals were used as received.

Oilseed material. The rape- and sunflowerseed used in this study were purchased from a retail store located in Lleida (Catalonia, Spain). The seed was milled using a standard coffee grinder before use.

Preparation of standards. Vegetable oils used as standards for HPLC analysis were obtained from the oilseeds by previously established methods (10). FFA and FAME were also obtained from the corresponding vegetable oil by standard methods.

Preparation of resting cells. Aspergillus flavus CECT 20475 was cultivated in a synthetic liquid medium that contained 2 g asparagine, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 5 mg thiamine hydrochloride, 1.45 mg $Fe(NO_3)_3 \cdot 9H_2O$, 0.88 mg $ZnSO_4 \cdot 7H_2O$, and 0.235 mg $MnSO_4 \cdot H_2O$ per liter in distilled water. The initial pH of the medium was adjusted to 5.5–6.0. The medium was sterilized (121°C for 15 min) in 250-mL aliquots, after which 1% (vol/vol) refined sunflower oil was added aseptically. The medium was inoculated with 2.5 mL of an *A. flavus* spore suspension ($1–4 \times 10^6$ spores mL^{-1}) and then incubated at 28°C for 5 d on an orbital shaker (200 rpm). Mycelium was harvested from the culture medium using a Büchner funnel and was washed with distilled water followed by acetone. The mycelium was then dried under vacuum for 18 h and ground to a powder.

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Reactive extraction using a solvent. Three grams of ground oilseeds and 0.3 g of resting cells were added to a 50-mL screw-capped bottle. The mixture was suspended in isooctane or hexane and agitated on an orbital shaker at 200 rpm and 50°C throughout the experimental period. An appropriate amount of water and/or methanol was added in due time. Five hundred microliter samples were withdrawn every 24 h and filtered. One hundred microliters of the filtered samples was evaporated to constant weight. The crude extract was then redissolved in 0.4 mL of acetone/acetonitrile (1:1 vol/vol) and analyzed by HPLC, as described below. Two hundred microliters also was dissolved in 4 mL of ethanol/diethyl ether (1:1 vol/vol) solution and titrated using 0.01 M NaOH solution.

Reactive extraction without solvent. Three grams of ground oilseeds and 0.3 g of resting cells were mixed using a standard coffee grinder. The solid mixture was then added to a 50-mL screw-capped bottle and agitated on an orbital shaker at 200 rpm and 50°C for 96 h. Water and/or methanol was added in due time. The mixture was finally suspended in 25 mL of hexane and agitated on an orbital shaker at 200 rpm and 50°C for 30 min. The resulting organic solution was recovered by filtration, and the solid was re-extracted as before. The organic solutions were combined and evaporated. Aliquots of the crude extract were dissolved and analyzed as described above. Control experiments were carried out to determine the possible effect of the extraction path. Blank experiments also were carried out without resting cells as biocatalyst.

HPLC analysis. HPLC analyses were carried out with a Waters Series 600 pumping system, a Waters 710 autosampler, and a Waters 2690 UV detector at 210 nm (Waters Cromatografia SA, Barcelona, Spain). Ten microliters of each sample was injected into a Simmetry C_{18} 5 µm (150 × 3.9 mm) (Waters Cromatografia SA) reversed-phase column at 40°C. The solvents used were acetonitrile/0.1% trifluoroacetic acid aqueous solution (95:5 vol/vol) (solvent A) and acetone (solvent B). The gradient program was started at 100% of A at a flow rate of 0.7 mL/min. After 5 min the flow rate was increased at 1 mL/min and the gradient was ramped to 50% B at 5 mL/min. Finally, the flow rate was increased to 1.5 mL/min and held for 15 min. Chromatographic peaks were recorded and integrated using Millenium 32 computer software (Waters Cromatographia SA).

Determination of percentages of recovered crude extracts. Percentages were determined considering the initial content of TAG in the oilseeds and the different M.W. of the FA and FAME produced in each experiment.

RESULTS AND DISCUSSION

An initial study was carried out to investigate the capacity of the proposed method. This was conducted by mixing rapeseed (3 g), the resting cells (0.3 g), and methanol (0.5 mL) in isooctane (25 mL) (Fig. 1). The formation of FAME already was apparent after 24 h of reaction, although the yield was

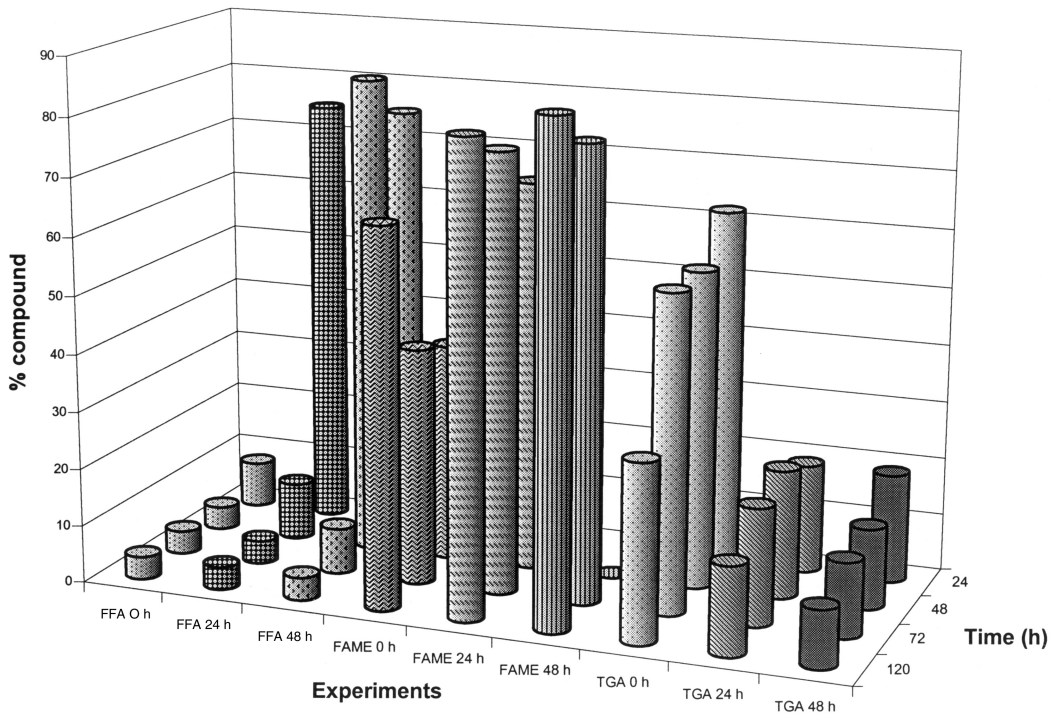

FIG. 1. Percentage of FFA, FAME, and TAG obtained when 3.0 g of ground rapeseed and 0.3 g of *Aspergillus flavus* resting cells were suspended in 25 mL of isooctane and shaken at 50°C. Water (0.5 mL) was added at 24 h. Methanol (0.5 mL) was added at 0, 24, and 48 h, respectively, as indicated on the *x*-axis (Experiments). Percentages were determined by considering the weight of crude extract.

TABLE 1
FA Recovered as a Fraction of the Amount Present in the Oilseed Before Extraction[a]

Experiment	% FA recovered	Total reaction time (h)
A0	96	120
A24	100	120
A48	104	120
B	87	96
C	100	96

[a]Ground rapeseed (3.0 g) and *Aspergillus flavus* resting cells (0.3 g) were suspended in 25 mL of solvent and shaken at 50°C. Experiment A0: Isooctane was used, and 0.5 mL of methanol was added at 0 h. A24: Isooctane was used, and 0.5 mL methanol was added at 24 h. A48: Isooctane was used, and 0.5 mL methanol was added at 48 h. B: Isooctane was used, and 0.5 mL water, 0.2 mL methanol, and 0.1 mL methanol were added at 24, 48, and 72 h, respectively. C: Hexane was used, and 0.5 mL water, 0.2 mL methanol, and 0.1 mL methanol were added at 24, 48, and 72 h, respectively. Percentages were determined considering the content of TAG in the rapeseed oil.

only 25%. However, the TAG yield was 62% and the FFA 8%. After 120 h, the amount of FA recovered relative to that in the seeds was nearly 100% of that expected (Table 1). At this point, the yield of FAME rose to 65%, whereas the percentages of TAG and FFA fell to 31 and 4%, respectively. Despite the moderate yield reflected by these results, the possibility that methanol could inactivate the biocatalyst (12) led us to study other approaches.

Figure 1 shows the effect of adding methanol (0.5 mL; threefold molar excess) 24 and 48 h after the reactive extraction had started. After 120 h of reaction, the amount of FAME was clearly higher, in both cases, than when all methanol was added at the start of the reaction. Moreover, the TAG percentage was less than 20% in both experiments. These results agree with others reported by several authors (13–15), which indicated that methanol inactivation could be overridden by a sequential addition of this reagent. Surprisingly, TAG amounts were also very low after 24 h of reactive extraction, as observed from the presence of large quantities of FFA. In comparing the three reactions, it seems evident that TAG hydrolysis and the subsequent esterification of FFA is faster than the transesterification itself. These results confirm those reported by Kaieda *et al.* (16) using an extracellular lipase from *Rhizopus oryzae*. Furthermore, in both cases of sequential methanol addition the yield of FA was nearly 100% of the theoretical value (Table 1).

In an attempt to increase the TAG transformation, an experiment was designed to increase TAG hydrolysis and reduce putative methanol enzyme inactivation. Hexane, a hydrocarbon with a lower log *P* (where *P* represents hydrophobicity) than isooctane (17), was investigated to assist the hydrolysis. Figure 2 shows the results when 0.5 mL of water was added 24 h after the start of incubation. Methanol was added in two portions (0.2 and 0.1 mL) at 48 and 72 h. No TAG were now detected after 24 h of reaction; yields of FFA were 94 (isooctane) and 96% (hexane). These yields rose to 96 (isooctane) and 97% (hexane) after 48 h of reaction. The successive addition of methanol led to a 94% yield of FAME, with a 4% residual FFA yield when isooctane was used. The reactive extraction carried out in hexane rendered a crude extract containing 90% of

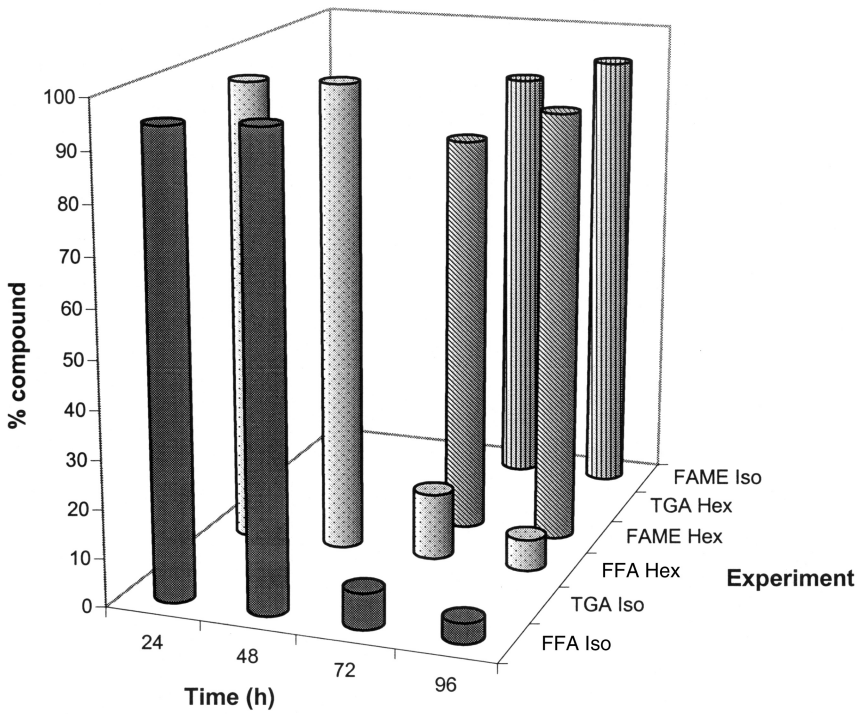

FIG. 2. Percentage of FFA, FAME, and TAG obtained when 3.0 g of ground rapeseed and 0.3 g of *A. flavus* resting cells were suspended in 25 mL of either isooctane (Iso) or hexane (Hex) and shaken at 50°C. Methanol (0.2 and 0.1 mL) was added at 48 and 72 h, respectively. Percentages were determined by considering the weight of crude extract.

TABLE 2
Percentage of FFA, FAME, TAG, and Total FA Recovery from Ground Rapeseed and *A. flavus* Resting Cells[a]

Hexane (mL)	% TAG	% FFA	% FAME	% Recovered FA	Time (h)
12	ND	8	90	88	96
0	ND	8	89	62	96
0[b]	33	55	9	23	96
25[c]	ND	20	77	37	1

[a]Ground rapeseed (3.0 g) and *A. flavus* resting cells (0.3 g) were suspended in various amounts of hexane and shaken at 50°C. ND, not detected. For other abbreviation see Table 1.
[b]Blank experiment: No resting cells were used.
[c]Control experiment: All the materials were mixed together at the start. Percentages were determined as described in Figure 1 and Table 1.

TABLE 3
Percentage of FFA, FAME, TAG, and Total FA Recovery from Ground Sunflower Seed and *A. flavus* Resting Cells[a]

Hexane (mL)	% TAG	% FFA	% FAME	% Recovered FA	Time (h)
0	1	6	92	90	96
0[b]	98	ND	ND	73	96
25[c]	2	23	70	55	1

[a]Ground sunflowerseed (3.0 g) and *A. flavus* resting cells (0.3 g) were shaken at 50°C.
[b]Blank experiment: No resting cells were used.
[c]Control experiment: All the materials were mixed together at the start. Percentages were determined as described in Figure 1 and Table 1. For abbreviations see Tables 1 and 2.

FAME and 7% of FFA. However, the crude extract yield from isooctane now dropped to 87%, whereas it was 100% when hexane was used (Table 1). Nonetheless, these differences in the yield in the final crude extract could be a consequence of the heterogeneous nature of the initial material.

Once the capability of the designed method was manifest, we studied the possibility of reducing the amount of solvent. Table 2 shows that when the amount of hexane was reduced by half, the recovery of FA was 88% of maximum theoretical. The crude extract contained 94% FAME and 4% FFA. Note that reactive extraction without solvent recovered 62% of the FA, being 89% FAME and 8% FFA.

Two new experiments were carried out to check whether this last result was solely a consequence of the final extractive period or of the rapeseed's own putative lipases (18). The first one consisted of mixing all the materials together for 60 min; in this case 37% of the FA were recovered, consisting of 77% FAME and 20% FFA. In the second experiment, no resting cells were introduced. After 96 h of reaction a 23% FA yield was achieved, being 55% FFA, 33% TAG, and only 9% FAME. It therefore seems evident that resting cells are necessary and that at least part of the process is carried out in the absence of solvent.

Several examples have demonstrated the possibility of enzymes working in solvent-free systems (19), although most of them involve the use of liquid substrates (15,20). Nevertheless, the moderate final yields (62% of theoretical FA recovery) lead us to suppose that resting cells might only transform the TAG that are released from the damaged rapeseed. Two experiments were therefore carried out using soft oilseeds such as sunflowerseed. Table 3 shows 90 and 73% yields, on a total FA basis, in the presence and absence of resting cells, respectively. The presence of resting cells led to a preparation containing 92% FAME 6% FFA, and 1% of remaining TAG. On the other hand, experiments without resting cells led to a crude extract with 98% TAG and no detected FFA or FAME. Thus, sunflowerseed did not seem to contain any active carboxyesterase, unlike rapeseed. Finally, a 55% yield was obtained when all materials were mixed together for 60 min. This experiment led to a product with 2% TAG, 23% FFA, and 70% FAME. Thus, the described method is potentially more versatile than the one proposed by Rüsch gen. Klass and Warwel (9).

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