Optimization of Fatty Acid Extraction from *Phaeodactylum tricornutum* UTEX 640 Biomass

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ABSTRACT: Fatty acids in the microalga *Phaeodactylum tricornutum* were isolated using an optimized three-step method: extraction of crude fatty acid potassium salts made by direct saponification of lipids in the microalgal biomass with KOH/ethanol (96%, vol/vol), separation of unsaponifiable lipids by extraction with hexane, and final purification of fatty acids by acidification of the alcoholic solution of potassium soaps followed by extraction of fatty acid into hexane. Direct saponification was carried out in ethanol (96%, vol/vol) using 2.09 mL ethanol (96%) per gram of wet biomass (10 mL/g of dry biomass) mixed with 0.4 g KOH/g of biomass. Under these conditions the fatty acid yield was 87%. The optimal water content of the alcoholic solution for extraction of the unsapononifiables was established as 40%, w/w. Data on equilibrium carotenoid distribution between the alcoholic (40%, w/w water) and hexane phases were determined. These data allow prediction of the carotenoid yields with different volumes of hexane in several extraction steps. The optimal pH of the alcoholic solution before extracting the purified fatty acid was established as pH 6, and the equilibrium fatty acid distribution between the alcoholic and hexane phases was determined. This optimized method permited a 20% reduction in the production costs of highly purified eicosapentaenoic acid (EPA) in the three-step preparative process (extraction of fatty acid, concentration of polyunsaturated fatty acids by the urea method, and EPA fractionation through preparative high-performance liquid chromatography) previously developed by the authors.
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KEY WORDS: Eicosapentaenoic acid (EPA), fatty acid extraction, fatty acid purification, *Phaeodactylum tricornutum.*

Technology has been evaluated for the potential production of polyunsaturated fatty acids (PUFA) by microorganisms (1). Human diets formulated to contain PUFA reportedly produce a preventive/regulatory effect in the treatment of circulatory system diseases (2). Today's technology has made oils from microalgae competitive with those from fish as a source of these raw materials (3). Under certain growth conditions, the microalga *Phaeodactylum tricornutum* has a high rate of eicosapentaenoic acid (EPA, 20:5n-3) production. EPA is a fatty acid essential for human metabolism and is involved in blood lipids equilibrium. EPA lowers triglyceride levels in blood serum, reduces the degree of platelet aggregation, is anti-inflammatory (4), and prevents hypertriglyceridemia (5) and various carcinomas (6). Total *Phaeodactylum tricornutum* fatty acids include 20–40%, w/w, EPA and small amounts of other PUFA that are difficult to separate from EPA during the purification process (7,8).

Current processes cannot provide the highly purified products required by the pharmaceutical industry for preclinical and clinical trials (2). Therefore, further development in this area of microalgal biotechnology will require rapid reliable methods of PUFA extraction and purification from the microalgal biomass. At the same time, such treatments must minimize autooxidative degradation and the presence of artifacts. Furthermore, solvents used to extract PUFA for the pharmacological industry must be selected bearing in mind their toxicity, handling, safety, and cost.

A three-step process for obtaining highly purified PUFA from cod liver oil and microalgae has recently been developed. The three steps are, fatty acid extraction by direct saponification of biomass, enrichments of PUFA by urea fractionation, and isolation of PUFA through preparative high-performance liquid chromatography (HPLC). In this way, 96%-pure EPA and 95%-pure docosahexaenoic acid (DHA) from the microalga *Isochrysis galbana* (9), 96%-pure EPA from *P. tricornutum* (8,10), and 94%-pure EPA and 81%-pure arachidonic acid (AA) from *Porphyridium cruentum* (11) have been obtained. A preliminary estimate of the cost of obtaining EPA from wet *P. tricornutum* biomass by this procedure puts the production cost of 96%-pure EPA at US$188 per gram, extraction by direct saponification of biomass and purification through preparative HPLC being the most expensive steps (67% of the total costs) (10). The objective of this work was to reduce extraction costs by optimizing the first step (the extraction of fatty acid from biomass).

MATERIALS AND METHODS

Microalgal biomass. Wet biomass of the marine microalga *P. tricornutum* UTEX 640 was used as an oil-rich substrate with a high proportion of EPA. Cells were grown in an outdoor tubular photobioreactor, harvested by centrifugation at

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1800 × *g*, and then stored at −18°C until use. The wet paste biomass contained 20.9 ± 1.3% dry biomass. The total fatty acid content in the biomass was 10.9 ± 0.5% of the dry weight, and the EPA content was 26% of the total fatty acids.

Fatty acid extraction from biomass. Fatty acid extraction was performed by the three-step method shown in Figure 1, that is, direct saponification of wet biomass, followed by extraction of unsaponifiables and extraction of purified fatty acids.

Direct saponification of wet biomass. In a typical experiment 4.8 g of wet biomass (1 g of dry biomass) was treated with 76 mL of ethanol (96%, vol/vol), containing 1.6 g of KOH (85% pure), in a 2.5-L reactor that was jacketed for temperature control. Saponification was carried out at 60°C for 1 h with constant agitation in an argon atmosphere. The mixture obtained was then filtered through a 100–160 µm microporous glass filter, and the biomass residue was washed with 20 mL of ethanol (96%).

Extraction of unsaponifiables. In a typical experiment 20 mL of water was added to 96 mL of the soap solution (11% w/w water and 47 ± 2 mg/L of carotenoids) to obtain a solution with 28% w/w water, and unsaponifiables were extracted in subdued light at 20°C by adding hexane and shaking. The two phases were then separated and two aliquots of each phase taken for carotenoid determination. To study equilibrium carotenoid distribution between the alcoholic phase and hexane, different volumes of hexane were added to 25 mL of a 40% w/w, water/alcoholic solution (34.0 ± 1.1 mg/L carotenoids). These extractions were carried out at 20 and 40°C. Unsaponifiables were also separated from the soap solution in several extraction steps with different volumes of hexane.

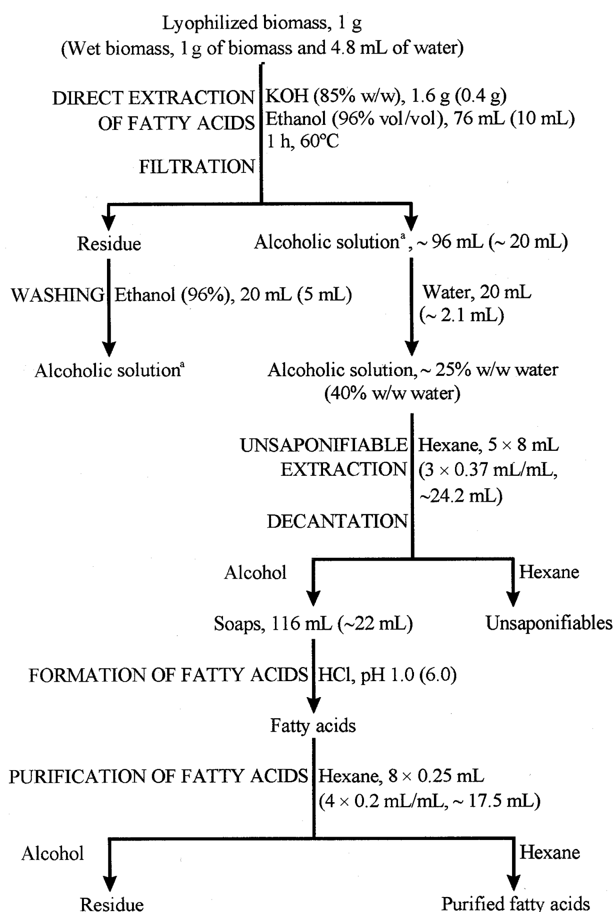

FIG. 1. Original and optimized (in parentheses) process to extract and purify fatty acids from the microalga *Phaeodactylum tricornutum.* ([a]The two alcoholic solutions are mixed.)

Extraction of purified fatty acids. In a typical experiment the pH of the 40% w/w water/alcoholic solution was adjusted to pH 1.0 using 35% HCl. The fatty acid concentration of this solution was 903 ± 18 mg/L. The equilibrium distribution of fatty acids was determined by adding different volumes of hexane to 25 mL of fatty acid solution. Extractions were performed in subdued light in an argon atmosphere at 20°C with agitation. The phases were separated, and an aliquot of each was taken for fatty acid determination. Extraction of fatty acid was also carried out in several steps with different volumes of hexane (Fig. 1).

Carotenoid determination. Carotenoids were determined as a measure of the unsaponifiable extraction yields using a modified version of the method employed by Whyte (12). To determine carotenoids in the alcoholic solution a known volume of this solution was dried under a N_2 stream. The residue was resuspended in an aqueous solution with 60% KOH, and the carotenoids were extracted with ethyl ether. Then the ethyl ether phase was dried, and carotenoids were resuspended in a known volume of acetone. To determine carotenoids in the hexane phase a known volume of this solution was dried, and the residue was resuspended in a known volume of acetone. The optical densities (OD) of these suspensions were determined at 444 nm using acetone as the reference. Carotenoids concentration (C) was determined by Equation 1:

$$C\left(\text{mg L}^{-1}\right) = 4.32\ \text{OD} - 0.0439 \qquad [1]$$

This equation was obtained using β-carotene as the solute and acetone as the solvent with a correlation coefficient (r^2) of 0.9998. Equation 1 was used to determine carotenoids in the initial alcoholic solution and in hexane upon reaching equilibrium. Carotenoids in the alcoholic phases were determined by difference between the amount of initial carotenoids and the amount in the hexane phase.

Fatty acid determination. Fatty acids in feedstock, hexane, and alcoholic phases were analyzed by capillary gas chromatography (GC). To determine fatty acids in the hexane phase, a known volume was dried in a N_2 stream, and methylation was by direct transesterification following the method of Lepage and Roy (13). Methylation and methyl ester analysis have been described elsewhere (14). To determine fatty acids in the alcoholic phase, a known volume was mixed with an equal volume of water, and HCl was added to pH 1.0; fatty acids were extracted three times with hexane, and the amount in hexane was then analyzed as indicated above. Fatty acid yields were then calculated (14). The total fatty acid content in biomass was determined by direct transesterification of fatty acid in biomass.

Protein and chlorophyll determination. Proteins were determined in the final alcoholic solution after separating the purified fatty acids, as described by Lowry *et al*. (15). Chlorophylls in the biomass were measured according to the method of Hansmann (16).

RESULTS AND DISCUSSION

Fatty acid extraction from biomass. In earlier work several low-toxicity solvents were evaluated for extracting fatty acids from the microalgae *I. galbana* (14) and *P. tricornutum* (8). The best solvents were ethanol (96% vol/vol) and hexane/ethanol (96%) (1:2.5, vol/vol). However, lyophilized biomass was extracted and large volumes of solvents were used (Fig. 1). Table 1 shows that fatty acid yield decreased about 4% when the ratio of ethanol (96%)/wet biomass decreased from 20 to 1 mL/g, and the ethanol concentration decreased from 92 to 55% (vol/vol), as consequence of water present in the wet biomass. Therefore, an ethanol (96%)/wet biomass ratio of 1.05 mL/g, which is much lower than what was used previously (76 mL/g of lyophilized biomass) (8), seems to be enough. However, 2.09 mL of ethanol (96%) per gram of wet biomass (10 mL/g of dry biomass) was used, because the subsequent filtration took too long with an ethanol (96%)/wet biomass ratio of 1.05 mL/g. The partial yields of EPA were similar to the overall fatty acid yields (Table 1). When the extraction was made from lyophilized biomass a fatty acid yield of 96.2% was obtained (Table 1). The decrease in the fatty acid extraction yield obtained with wet biomass can be compensated by the decrease in cost because the lyophilization is omitted.

Extraction of unsaponifiables. The crude fatty acid extract obtained with ethanol (96%)/KOH (i.e., the initial alcoholic solution in Fig. 1) contains the potassium salts of fatty acids, pigments such as carotenoids and chlorophylls (the chlorophylls in *P. tricornutum* biomass were about 1% by dry weight), proteins (the protein concentration in the final alcoholic solution was 14.8 g/L) and other lipid and nonlipid contaminants. The unsaponifiable lipids, such as carotenoids, can be extracted by treating the crude extract with apolar solvents such as chloroform or hexane, in which soaps are not soluble. Here hexane was used because it is less toxic than chloroform. The alcoholic solution contained a little water [i.e., only the water added with the wet biomass and ethanol (96% vol/vol)]. An increase in the water content (obtained by adding water to a solution with 11% w/w water and 45 mg/L of unsaponifiables as β-carotene decreased the miscibility of the alcoholic solution and hexane and increased the unsaponifiable extraction yield. At water contents of 11, 30, 40, 50, 60, and 70% w/w, the unsaponifiable extraction yields with respect to the amount of unsaponifiables contained in the alcohol solution were 15.3, 24.6, 37.6, 50.3, 60.1, and 54.6% w/w of β-carotene, respectively (extraction carried out at 20°C and an alcohol solution/hexane ratio 1:1, vol/vol). At water percentages over 60%, emulsions formed, the stability of which increased with water content. Also, when the 50–60% water/alcoholic solutions were later acidified to extract the fatty acids (Fig. 1), emulsions again formed. Emulsions make extraction difficult and decrease the fatty acid recovery. So an optimal water content of 40% was chosen to prevent emulsions formation. In a previous article (8), it was reported that emulsions formed more easily when the biomass residue was not separated from the soap solution by filtration and when this residue was washed with water instead of ethanol. The alcoholic solution contained surfactant agents, such as soaps and proteins, that facilitated and stabilized the alcoholic/hexane emulsion. An increase in the water content increased the amount of hydrating water that stabilized the hydrophilic moieties of the surfactant agents, thus stabilizing the emulsion.

To optimize the hexane/alcoholic solution ratio used for extracting the unsaponifiables, we determined the equilibrium carotenoid distribution between the hexane and alcoholic (40% w/w water) phases and/or the relationship between the extraction yield and the hexane/alcohol ratio (Fig. 2, Table 2). Table 2 shows that the recovery of unsaponifiables with hexane increased with the hexane/alcohol phase ratio. However, a large volume of hexane was required to extract the unsaponifiables quantitatively. The equilibrium distribution constant values (Table 2) and the equilibrium data (Fig. 2) also show that equilibrium was greatly displaced to the alcoholic phase and that when the temperature was increased from 20 to 40°C, the equilibrium was only slightly displaced to the hexane phase. The equilibrium data enable prediction of the unsaponifiable yield when the extraction is done in one or several steps with different volumes of hexane. Table 3 compares the extraction yields obtained experimentally (by a five-step hexane extraction) and those obtained from the equilibrium data. The calculation of the latter is based on a sequence of five batch extraction steps, typical in the chemical process industry, assuming that equilibrium has been attained in each

TABLE 1
Influence of Ethanol (96%)/Wet Biomass (20.9 ± 1.3% dry biomass) Ratio on the Fatty Acid Yield Obtained by Direct Saponification of Wet *Phaeodactylum tricornutum* Biomass

Ethanol (96%)/ wet biomass (mL/g)	Ethanol (96%/ dry biomass (mL/g)	Ethanol[a] (%) (vol/vol)	Fatty acid yield[b] (%)	EPA yield (%)
1.05	5	55	86.0	86.0
2.09	10	70	87.0	90.2
2.93	14	76	86.3	89.7
3.97	19	80	86.9	88.5
7.94	38	87	88.5	89.2
11.91	57	90	90.6	90.9
18.88	76	92	90.4	92.1
19.86	95	92	90.0	92.2
—	76[c]	96	96.2[d]	98.3[d]

[a]The concentration of ethanol diminishes as a result of the water contained in the wet biomass.
[b]Fatty acids were extracted by four aliquots of 0.2 mL hexane per mL of alcohol solution at 20°C. Percentage of fatty acid extracted with respect to the total amount of fatty acids contained in the initial biomass.
[c]Ethanol/dry biomass ratio corresponds to the usual solvent/biomass ratio used by the method of Bligh and Dyer (Ref. 18).
[d]These yields were obtained using lyophilized biomass.

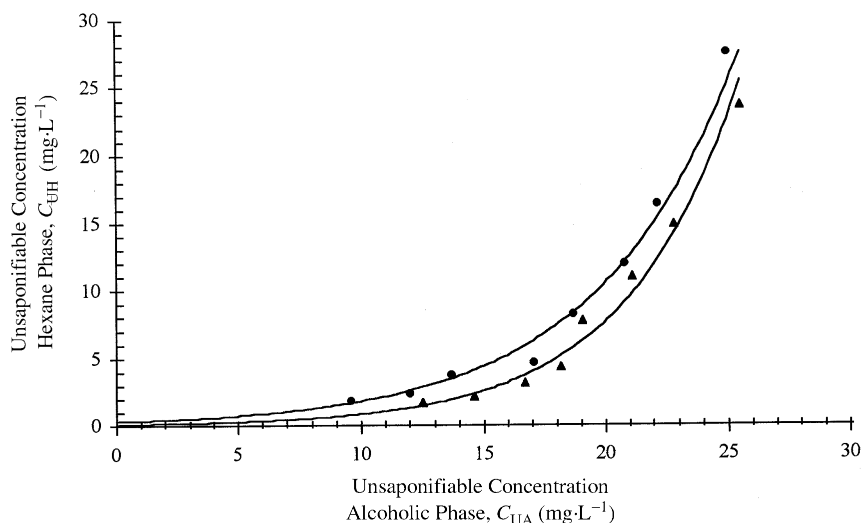

FIG. 2. Equilibrium distribution of unsaponifiables between the alcoholic (40% w/w water) phase and hexane at 20 (▲) and 40°C (●). The experimental data were fit by the equations C_{UH} = 0.10 exp (0.217 C_{UA}) at 20°C and C_{UH} = 0.32 exp (0.175 C_{UA}) at 40°C. The correlation coefficients (r^2) were 0.9757 and 0.9797, respectively. C_{UH} is the concentration of unsaponifiables in the hexane phase (mg·L^{-1}) and C_{UA} is the concentration of unsaponifiables in the alcoholic phase. The mean deviations between the experimental C_{UH} and the values predicted by the equations are 12.5 and 9.5% of the experimental C_{UH} at 20 and 40°C, respectively.

step and further that the two phases are totally immiscible (17). Good correlation is observed (less than 10% difference). Table 3 shows that maximal unsaponifiable yields of 48.7% were obtained with a total hexane/alcohol solution ratio of 1.87 vol/vol in five extraction steps. Some unsaponifiables not extracted will remain in the final fatty acid extract, although unsaponifiable recovery here (48.7%) is better than in the original procedure (38%, Fig. 1) because the extraction is from an alcoholic phase with a lower water content (approximately 25% w/w water, Fig. 1). Depending on the intended use of the final fatty acid extract, more extractions of unsaponifiables may be necessary.

Extraction of purified fatty acids. In early work (8) the pH of the alcoholic potassium salt solution was adjusted to 1 before extracting the purified fatty acids. However in some experiments carried out using the optimized conditions, emulsions formed when the pH was lowered to this value and hexane was added. At this point, solubility of surfactant agents such as proteins contained in the alcoholic solution may decrease with the change in pH. Even small amounts of these surfactant agents can accumulate in the alcoholic solu-

TABLE 2
Influence of the Hexane/Alcohol Solution Ratio (H/A) on the Unsaponifiable Extraction Yields (R_U) and Equilibrium Distribution Constants (K_U) of Unsaponifiables Between the Hexane and the Alcoholic Solution

H/A (vol/vol)	Extraction temperature 20°C		Extraction temperature 40°C	
	K_U[a]	R_U[b] (% w/w of β-carotene)	K_U[a]	R_U[b] (% w/w of β-carotene)
80.0	—	100	—	—
40.0	5.43	99.7	—	98.9
14.0	0.14	71.7	0.20	79.8
10.0	0.15	64.9	0.21	73.0
6.0	0.19	56.1	0.28	66.3
4.0	0.24	50.8	0.27	55.1
2.0	0.41	45.7	0.44	47.7
1.2	0.52	38.9	0.58	40.9
0.8	0.65	33.4	0.74	36.0
0.4	0.93	25.1	1.10	26.6

[a]$K_U = C_{UH}/C_{UA}$, where C_{UH} is the concentration of unsaponifiables in the hexane phase and C_{UA} is the concentration of unsaponifiables in the alcoholic phase.
[b]Percentage of unsaponifiables extracted with respect to the amount of unsaponifiables contained in the initial alcoholic solution.

TABLE 3
Comparison Between the Yields of Unsaponifiables Obtained by Extraction with Hexane and the Yield Predicted by the Equilibrium Data

Extraction[a] step	Unsaponifiable yield (% w/w of β-carotene)		
	Step[b]	ΣStep[c]	Equilibrium data[d]
1	26.0	26.0	29.1
2	9.0	35.0	40.0
3	6.3	41.3	45.6
4	4.3	45.6	50.0
5	3.1	48.7	53.5

[a]Extractions were made at 20°C using a hexane/alcohol solution (40% w/w water) ratio (H/A) of 0.37 vol/vol per step.
[b]Unsaponifiable yield obtained experimentally. Percentage of unsaponifiables extracted with respect to the amount of unsaponifiables contained in the alcohol solution.
[c]Unsaponifiable yield obtained experimentally. Percentage of unsaponifiables extracted with respect to the amount of unsaponifiables contained in the initial alcohol solution.
[d]Unsaponifiable yield obtained from the equilibrium data presented in Figure 2 [C_{UH} = 0.10 exp (0.217 C_{UA})]. For abbreviations see Table 2.

TABLE 4
Influence of the Hexane/Alcohol Solution Ratio on the Fatty Acid Yields and Distribution Constants of Fatty Acids and Eicosapentaenoic Acid[a]

H/A (vol/vol)	K_F	K_{EPA}	R_F (%)
14.0	—	—	100
10.0	—	—	99.4
6.0	—	—	98.7
4.0	17.8	18.5	98.3
2.0	16.2	16.9	97.1
1.2	15.0	15.8	97.4
0.8	15.9	16.8	92.6
0.4	18.5	17.8	83.6
0.2	26.1	25.0	81.7

[a]Extraction temperature, 20°C. H/A = hexane/alcohol solution ratio. $K_F = C_{FH}/C_{FA}$, where C_{FH} is the fatty acid concentration in the hexane phase and C_{FA} is the fatty acid concentration in the alcohol phase. K_{EPA} = distribution constant for eicosapentaenoic acid (EPA) concentration in the hexane phase/EPA concentration in the alcoholic phase. R_F = percentage of fatty acids extracted with respect to the amount of fatty acids contained in the alcoholic solution.

TABLE 5
Comparison Between the Fatty Acid and EPA Yields Obtained by Hexane Extraction and Predicted by the Equilibrium Data

	Fatty acid yield (%)			EPA yield (%)		
Extraction[a] step	Step[b]	ΣStep[c]	Equilibrium data[d]	Step[b]	ΣStep[c]	Equilibrium data[d]
1	78.6	78.6	81.0	79.2	79.2	83.7
2	11.2	89.8	96.7	11.8	91.0	96.8
3	4.7	94.5	99.4	4.7	95.6	99.3
4	1.9	96.4	99.9	1.7	97.4	99.8

[a]Extractions were made using a hexane/alcohol solution (40% w/w water) ratio of 0.20 vol/vol per step.
[b]Fatty acid (or EPA) yield obtained experimentally, expressed as percentage of fatty acids (or EPA) extracted with respect to the amount of fatty acids (or EPA) contained in the alcoholic solution.
[c]Fatty acid (or EPA) yield obtained experimentally, expressed as percentage of fatty acids (or EPA) extracted with respect to the amount of fatty acids (or EPA) contained in the initial alcohol solution.
[d]Fatty acid (or EPA) yields obtained from the equilibrium data presented in Figure 3; [$C_{FH} = 261.6 \exp(0.0174\ C_{FA})$]. For abbreviations see Tables 2 and 4.

tion/hexane interface, hence stabilizing the emulsion. Thus, a study of the influence of pH on the fatty acid yield was carried out. Compared to the fatty acid and EPA yields obtained at pH 1, fatty acid and EPA yields did not decrease when pH was adjusted between 3 and 6. At pH 7, low fatty acid and EPA extraction yields were obtained presumably because the fatty acids were still partially in the form of potassium salts.

The equilibrium distribution of fatty acids between the hexane and alcoholic phases was similar to the equilibrium distribution of unsaponifiables. Table 4 shows that the recovery of fatty acids with hexane increases with the hexane/alcohol solution ratio. In this case, a quantitative extraction of fatty acids is possible using relatively low hexane/alcohol phase ratios. The EPA distribution constants are similar to those of the overall fatty acids, so similar EPA yields can be obtained. The equilibrium data represented in Figure 3 enable prediction of the fatty acid yields when the extraction is done in one or several steps with different volumes of hexane. Table 5 compares the extraction yields obtained experimentally (by a four-step hexane extraction) to those based on a sequence of four batch extraction steps, assuming that equilibrium is reached in each step and that the two phases are totally immiscible (17). Good correlation between the two yields may be observed (less than 4% difference).

Comparison of the original and optimized process. The optimized conditions identified in this work were: 10 mL

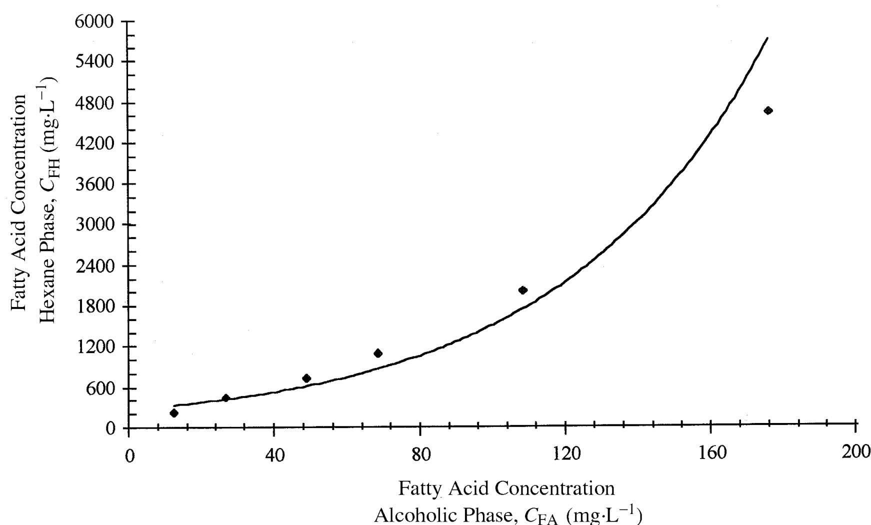

FIG. 3. Equilibrium distribution of fatty acids between the alcoholic (40% w/w water) phase and hexane at 20°C. The experimental data were fit by the equation $C_{FH} = 261.6 \exp(0.0174\ C_{FA})$, with a correlation coefficient (r^2) of 0.9501. C_{FH} is the fatty acid concentration in the hexane phase (mg·L^{-1}) and C_{FA} is the fatty acid concentration in the alcoholic phase; the mean deviation between the experimental C_{FH} and the values predicted by the equation is 20% of the experimental C_{FH}.

ethanol (96% vol/vol)/g biomass (2.09 mL/g wet biomass); washing of the biomass residue with 5 mL ethanol (96%)/g biomass; extraction of unsaponifiables by three extractions with 0.37 mL hexane/mL alcoholic solution (40% water w/w) each; and extraction of fatty acids by four extractions with 0.2 mL hexane/mL alcoholic solution (pH 6). The fatty acid yield was 87%, which was lower than the yield obtained in the original process (96.2%) (10). This is mainly because wet biomass was used instead of lyophilized biomass (see the last line in Table 1); however, elimination of the cost of lyophilization justifies this lower fatty acid yield. Figure 1 compares both original and optimized processes (amount in parentheses). As observed above, an important reduction in the amounts of hexane (about 90%) and ethanol (96%) (about 84%) has been achieved. In the earlier process the costs of extraction and purification to obtain highly puriried EPA from *P. tricornutum* were estimated at about US$188 per gram of 96% pure EPA, which included culture, extraction, concentration, and purification (10). By only optimizing the fatty acid extraction step, this cost could be reduced by about 20% (the production cost would be US$150 per gram of highly purified EPA). The production cost of highly purified EPA from cod liver oil was estimated at about US$369 per gram (10). After similar optimization, the cost of production of EPA from cod liver oil could only be reduced by about 8%, because only the volume of hexane in the final purification of the fatty acid was reduced. The extraction of fatty acids from cod liver oil is quite different, as the first step is saponification and it is not necessary to extract unsaponifiables (9,14). Although this method has been optimized for the microalga *P. tricornutum*, it can be extended to other microalgae such as *I. galbana* or *P. cruentum* with hardly any modification.

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