

## **BIOPROCESSING OF OILS AND FATS TO HIGHER VALUE-ADDED PRODUCTS**

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### **ABSTRACT**

Fats and oils were once the primary sources of aliphatic carbon compounds used by industry. With the availability of inexpensive petroleum feedstocks, the consumption of these commodities has declined in most industrial applications. In addition, this situation has been exacerbated because of the large increase in world production of fats and oils without a similar increase in consumption. Moreover, health-related concerns continue to erode domestic and export markets for edible fats and oils, especially animal fats. Despite their ready availability and competitive price the domestic non-food use of fats and oils continues to decline in almost all applications. To reverse these trends, our laboratory is evaluating the application of biocatalysis and biomimicry (chemical reactions that mimic enzyme reactions) to fats and oils. The goal of the research is to expand current uses and identifying new uses of fats and oils in higher-value added applications. Particular areas of research in which we have developed expertise and continue to explore and develop include: the lipase-catalyzed synthesis of low-calorie and nutraceutical triacylglycerols; the biocatalytic oxygenation of fatty acids; and the microbial production of biodegradable polymers from fat and oil substrates.

### **LIPASE-CATALYZED SYNTHESIS OF STRUCTURED LOW-CALORIE TRIACYLGLYCEROLS**

Interesterification is one of the major reactions used by industry for the modification of natural fats and oils. In its simplest form, interesterification corresponds to an exchange of acyl residues between two triacylglycerols (TAGs), resulting in the formation of new TAGs that have chemical and physical properties distinct from the starting TAGs (1). Presently, the interesterification of TAGs is conducted either chemically or enzymatically. Chemical interesterifications are generally catalyzed by metal alkoxides, which are relatively inexpensive, readily available, and easy to use. Chemical interesterification produces TAGs that have a

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predetermined composition but random distribution of fatty acyl groups on the glycerol backbone and often are referred to as tailored lipids (TL). On the other hand, because of their unique specificities, the use of lipases allow for the design of TAGs with a predetermined composition and distribution of fatty acyl groups on the glycerol backbone (2). Accordingly, TAGs prepared by lipase-catalyzed interesterification are referred to as structured lipids (SL). Examples of lipase-catalyzed interesterification (or transesterification) reactions abound in the literature (3-5). However, the applications of lipase-catalyzed interesterification reactions on an industrial scale are limited by the high cost of lipases. Consequently, the use of plant biocatalysts may have advantages owing to their lower cost and ready availability in comparison to their microbial and animal counterparts (6).

One such plant biocatalyst is *Carica papaya* latex (CPL), which is known for containing the enzyme papain, a thiol protease with many industrial applications, e.g., as a meat tenderizer, contact lens cleaner, digestive aid, or bloodstain remover in detergents. This plant exudate, however, also exhibits a lipase activity, which several groups have characterized (7-10). Exploiting these CPL selectivities, we recently reported the synthesis of structured low-calorie TAGs by interesterification of hydrogenated soybean oil (HSO) with tributyrin (11).

Low-calorie structured lipids are characterized by a combination of short-chain ( $C_{2-4}$ ) and long-chain ( $C_{16-22}$ ) acyl residues into a single triacylglycerol structure. Interest in these types of lipids stems from the fact that they contain only 5 cal/g (12) compared with the 9 cal/g of natural fats and oils because of the lower caloric content of short-chain acyl residues ( $C_{2-4}$ ) compared to their long-chain ( $C_{16-22}$ ) counterparts. The most familiar class of these low-calorie type fats is the so-called SALATRIM™ family, which are obtained by chemical interesterification. These TL are a mixture of two types of triacylglycerol (TAG) structures; the first contain two short-chain and one long-chain acyl residues (SSL-TAG), and the second contain two long-chain and one short-chain acyl residues (LLS-TAG). By predetermining the fatty acid composition and ratio of these mixtures of short and long-chain triacylglycerols (SLCT), it is possible to produce a range of SLCT products useful in several food applications. For example, low-calorie SLCT are used as baking chips, coatings, dips, baked products, or as cocoa butter substitutes (13).

The CPL lipase-catalyzed interesterification of HSO with tributyrin gave satisfactory yields of short- and long-chain triacylglycerol (SLCT) product (11). On the other hand, the reaction between HSO and triacetin under the same conditions was severely limited with regard to the formation of SLCT in that only minor amounts (<1%) of the desired SLCT were formed after 24 hours of reaction (Scheme

1). Tributyrin is miscible with HSO and mixtures of the two form a monophasic medium in lipase-catalyzed reactions. In contrast, triacetin is immiscible in HSO forming a separate phase and hence these substrates are presented as a biphasic medium in enzyme reactions. It was determined that the immiscibility of substrates (HSO and triacetin or tripropionin) was the main reason for poor results obtained in the aforementioned reactions. Ester interchange is known to be limited in biphasic systems when there is poor interfacial surface area for the enzyme to react with the substrates. However, ultrasonication of the mixture prior to addition of the enzyme did not result in improved yields of SLCT. Further attempts at optimizing the dispersion of the two-phase triacetin-HSO reaction system failed to improve the yields of desired SLCT products. Addition of a moderately polar solvent, such as methyl t-butyl ether, to partially dissolve the substrates improved the yields to about 10%. This also indicated that physical incompatibility was a major factor for the low yields of SLCT in the triacetin-HSO interesterification reactions.

The immobilization of polar substrates onto macroporous materials, such as silica or diatomaceous earth, has been described recently as a way to improve the synthesis of various mono- and diesters of hydrophilic diols (14). Using this approach we found that the addition of silica-adsorbed triacetin preparations to melted HSO significantly improved the yields of SLCT compared to reactions without silica. The porous silica served to physically support the polar triacetin in the interesterification experiments with HSO and, thus, served as a "polar substrate reservoir". Mixtures were tested at various mole ratios of HSO to triacetin using an amount of silica equaling the amount of triacetin by weight. As expected, increasing concentrations of triacetin resulted in increased formation of SLCT products. The reaction mixture of HSO and triacetin/silica (1:1 mole ratio) showed no increase in viscosity compared to the HSO-triacetin melt without silica. However, mixtures of HSO and triacetin/silica became much more viscous at a mole ratio of 1:2, and almost gel-like at a mole ratio of 1:4. In other experiments, the mole ratio of triacetin to HSO was increased while keeping the amount of silica constant. From this series of experiments, it was determined that about one g of silica was needed to adsorb one g of triacetin. This same observation was noted when preparing the triacetin/silica mixtures. At a 1:1 wt ratio of silica:triacetin, the powder character of the silica does not change, whereas at ratios greater than 1:1 the silica particles are suspended in triacetin, with the non-adsorbed triacetin forming an outer phase resulting in a gel-like structure.

The chemically synthesized SLCT, a product of hydrogenated soybean oil, and acetic and propionic acid, used in this study as reference was a mixture of short-short-long chain (SST-TAG, 90 wt %) and long-long-short-chain (LLS-TAG, 10 wt %)

TAGs. The appearance of newly formed SSL- and LLS-TAG in the enzyme-catalyzed reactions reported herein were followed using a high performance liquid chromatographic method developed for the separation of neutral lipid and SLCT mixtures (15). The chromatographic data for the CPL-catalyzed product showed that the ratio of LLS- and SSL-TAG was different from that of the chemically synthesized material. For example, using a four-fold molar excess of triacetin over HSO gave a product mixture containing about 40% SSL-TAG, 25% LLS-TAG, and 35% unreacted HSO after 120 h of reaction. To prepare a SLCT of composition similar to that of the chemically synthesized material, a larger molar excess of triacetin was needed over HSO. This was accomplished by adding multiple doses of triacetin over the time course of the CPL-catalyzed interesterification reaction. The initial reaction was started using a twofold molar excess of triacetin over HSO and an equal weight of silica to triacetin. The amount of silica initially added to the medium was not increased with further additions of triacetin. Additional volumes of triacetin (2 molar equivalents) were added to the reaction after the reaction reached its apparent equilibrium as determined by HPLC. A final ratio of SSL- to LLS-TAG of 90:10, a ratio comparable to the chemically synthesized product, was obtained after three subsequent additions of triacetin (final HSO/triacetin mole ratio: 1:8), **Scheme 1**. An HPLC chromatogram showing the two isomeric classes of compounds that represent each of the two major SLCT products, SSL<sub>1</sub> & SSL<sub>2</sub> and LLS<sub>1</sub> & LLS<sub>2</sub>, is shown in **Figure 1**, (panel A1). For comparative purposes, the chromatogram of the chemically synthesized product also is shown in **Figure 1** (panel B1).

## CATALYTIC OXYGENATION OF UNSATURATED FATTY ACIDS

With one exception commercial fats and oils are composed of fatty acids that contain only double bond and ester functionality and for many non-food uses derivatization of a fat or oil to modify or increase its chemical functionality is required. The one major exception is castor oil, which contains the monohydroxy substituted fatty acid ricinoleic acid. The value that this hydroxy group imparts to castor oil is indicated by its market price, which is approximately three-fold higher than that of other common oils. Another important class of industrial products is epoxy oils that are obtained by the epoxidation of unsaturated vegetable oils. These derivatives are produced in excess of 200 million lbs. per annum in the US and are used mainly as stabilizer-plasticizers for PVC. Although the hydroxyl and epoxy functional oils themselves are used in a number of applications, much of their value derives from their ability to be chemically transformed to other functional materials. Thus, for example, the hydroxyl group can be reacted to form a sulfate,

endowing the fatty material with detergent properties, and the presence of the epoxide allows for easy crosslinking in plastics. Inexpensive ways of introducing oxygen into common fatty acids from US vegetable oils, e.g., soybean and cottonseed oils, in the form of hydroxy or epoxy functional groups have the potential to promote increasing utilization of these oils as industrial materials.

Our laboratory has an active research program designed to investigate novel methods for introducing oxygen into fats and oils. This research has revealed a number of promising avenues for the formation of oxygenated materials, **Figure 2**. We have previously reported our investigations on epoxidation of unsaturated fatty acids using the oxone method (16,17), and our use of the enzyme hydroperoxide lyase to produce intermediate length aldehydic materials (18). Here we will describe the results of our recent work to prepare fatty alcohol epoxides and  $\omega$ -substituted fatty acids from poly unsaturated fatty acids.

The starting point for the synthesis is the preparation of a fatty acid hydroperoxide. This was accomplished using soybean lipoxygenase (LOX). This enzyme catalyzes the addition of oxygen to linoleic acid to form 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, HPODE, **Figure 2**. LOX is a relatively unstable enzyme, and methods to immobilize this enzyme were sought in order to stabilize LOX and to allow for its recovery and reuse (19, 20). Methods were devised to promote the formation of HPODE in organic solvent (21), and to promote the formation of hydroperoxide in fatty acids such as those found in phospholipids and methyl esters (22).

After obtaining high yields of HPODE, methods of converting this material to useful chemical intermediates were sought. Any number of catalysts are capable of rearranging HPODE to alcohol epoxides, including strong acid and ferrous iron (23). However, what was desired was a catalyst that gives alcohol epoxy materials of specific structure, which limited the available catalysts to some enzymes and transition metal catalysts. A number of different metal catalysts were examined for their effect on the methyl ester of HPODE (Me-HPODE). The methyl ester was used because it was found that the free acid was non-reactive to these catalysts. It was also determined that very low water levels were required because water either inhibited the reaction or participated in the ring opening of the epoxide. In the rearrangement of Me-HPODE by titanium (IV) isopropoxide, the predominant methyl ester that was formed was the threo isomer, methyl 11(*R*), 12(*R*)-epoxy-13(*S*)-hydroxy-9(*Z*)-octadecenoate (24), whereas  $\text{Nb}(\text{OC}_2\text{H}_5)_5$  gave its erythro analogue, demonstrating that both catalysts selectively promoted the formation of an  $\alpha$ -epoxide. Thus these results demonstrate that the combination of a regio- and a

stereospecific LOX and a specific metal ion catalyst will produce a fatty alcohol epoxide of specific structure. This specificity can be used to design homogeneous industrial products.

HPODE also can be cleaved by the enzyme hydroperoxide lyase (HPLS) to an  $\omega$ -oxo-fatty acid and a short volatile fragment [25], Figure 3. For example, HPLS from microalgae, such as *Chlorella*, cleaves HPODE into the C<sub>13</sub>  $\omega$ -oxo-fatty acid and a C<sub>5</sub>-fragment (pentane) whereas HPLS from higher plants cleaves HPODE into a C<sub>12</sub>  $\omega$ -oxo fatty acid and a C<sub>6</sub> fragment (hexanal). The oxo fatty acids can be converted to their respective  $\omega$ -hydroxy and  $\omega$ -dicarboxylic acid derivatives by chemical or enzymatic methods. These materials have potential application in the production of surfactants, polyesters, and polyamids similar to nylon 13,13. Moreover, the presence of double bonds in these products allows for further derivatization or cross-linking. However, enzymatic methods are needed that can reduce or oxidize these intermediates in an environmentally safe and cost-effective manner compared with chemical procedures.

## **BIODEGRADABLE POLY( $\beta$ -HYDROXYALKANOATE) POLYMERS FROM TRIGLYCERIDES**

Poly(hydroxyalkanoates) (PHAs) are naturally occurring, optically active polyesters that accumulate in numerous bacteria as carbon and energy storage materials (26-28). In most cases, the polymers contain  $\beta$ -linked repeat units and possess the general structure shown in Scheme 2. The R group varies based on the bacterium and the carbon substrate from which the polymer was formed (29). Recently, there has been significant interest in the use of PHAs for biodegradable thermoplastics. Because they are viewed as "environmentally friendly", they are being studied as potential replacements for synthetic plastics in several applications. One major drawback to the use of these polymers is the cost involved in production. Generally, the cost to produce a given PHA polymer on an industrial scale is greater than for a comparable synthetic polymer. To make PHA production more economical, two avenues can be pursued: produce PHAs whose properties allow for their use in unique applications; and lower the production costs either by increasing polymer yields or by using less expensive substrates. The latter possibility (and to some extent the former) can be achieved by using agricultural triglycerides as carbon substrates.

It is known that several bacteria (primarily pseudomonads) produce medium-chain-length PHAs from fatty acids (30-32). However, only recently have intact triglycerides been considered as feedstocks for PHA production. Three bacterial species have been shown to produce PHA from triglycerides. These include *Aeromonas caviae*, which produced a copolymer of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from olive oil (33), *Pseudomonas aeruginosa*, which produced a complex PHA copolymer when grown on euphorbia or castor oil (35), and *Pseudomonas resinovorans*, which produced a PHA from tallow (34). In our laboratory we have investigated the use of *P. resinovorans* to synthesize unique PHAs from other triglyceride substrates.

Six triglyceride substrates (lard, butter oil, olive oil, high oleic acid sunflower oil, coconut oil, and soybean oil) were screened as potential substrates for PHA production. A two stage fermentation was used to increase the number of viable cells prior to transfer into the polymer production medium in hopes that increased PHA yields could be achieved. Each triglyceride, whether animal fat or vegetable oil, supported cell growth, Table 1. This indicated that the organism showed no significant preference towards fats (solids) or oils (liquids) as substrates for growth and polymer production. After 48 h in the polymer production medium the cells were viewed under a phase-contrast microscope for the presence of phase-bright inclusions, evidence of polymer production. *P. resinovorans* produced an MCL-PHA from each triglyceride. This was evident by the presence of one or more PHA granules per bacterium that, when visually inspected, appeared to constitute approximately 50% of the cell mass. The cells were harvested by centrifugation and the cellular biomass, and PHA content and yield were determined, Table 1. The average PHA content for all tested triglycerides was 45%, and the average PHA yield was about 1.5 g/L. Thus our two-stage fermentation system resulted in a 200% increase in PHA production compared to previously reported results (32). Repeat-unit composition analysis by gas chromatography of the tallow-derived PHA showed that the predominant monomers of the polymers were 3-hydroxyoctanoate and 3-hydroxydecanoate. The number-average molecular mass of the PHA-tallow was 87-93 kDa as determined by gel permeation chromatography. Similarly, *Ps. saccharophila*, which produces a high level of extracellular lipase activity [36], was shown to grow and produce PHA in medium containing tallow or coconut oil as a sole carbon source [37]. Transmission electron microscopic study of the tallow-grown *Ps. saccharophila* showed the presence of intracellular inclusion bodies characteristic of PHA-producing bacteria, Figure 4. Ashby and Foglia [34] further characterized the utilization of a series of agricultural lipids by *Ps. resinovorans* for cell growth and mcl-PHA production. Table 1 shows that the organism yielded cell biomass in the range of 2.4-4.1 cell-dry-weight (CDW) per liter (L) culture when grown with animal

fat or vegetable oil as a sole carbon source. The PHA contents of these cells ranged from 43-59% of CDW. Most significantly, repeat-unit composition analysis showed that the chemical structures of the pendant groups are highly dependent on the feedstock. When coconut oil which contains predominantly the saturated acyl chains, was supplied as a substrate, the resulting polymers have repeat-units with virtually no detectable double-bond functional group Table 2. On the other hand, soybean oil-derived PHA contains a high degree of unsaturation in the pendant groups, Table 2. In summary, we have demonstrated that *Ps. resinovorans* and *Ps. saccharophila* are able to grow and synthesize PHA by using the inexpensive agricultural fats and oils as substrate. Furthermore, manipulating the type of feedstock used in the fermentation can modify the chemical composition and thus the physical properties of these biodegradable polymers.

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**TABLE 1. Cell Dry Weights and Poly(hydroxyalkanoate) Polymer Content and Yields of *P. resinovorans* Grown on Triglyceride Substrates.**

Substrate	Cell Yield <sup>a</sup> (g/L)	PHA content <sup>b</sup> (% dry weight)	PHA yield <sup>c</sup> (g/L)
Control			
Oleic acid	3.8	43	1.9
Animal fats			
Tallow	3.0	39.8	2.1
Lard	3.6	47.4	1.7
Butter oil	3.6	47.0	1.7
Vegetable oils			
Olive	3.4	43.1	1.5
Sunflower (high oleic)	3.1	41.2	1.3
Coconut	3.8	51.0	1.9
Soybean	2.9	44.5	1.3
Averages <sup>d</sup>	3.3	44.9	1.5

<sup>a</sup>Cell dry weight average yields (n = 3).

<sup>b</sup>PHA per cell dry weight averages (n = 3).

<sup>c</sup>Calculated by multiplying the cell yield (g/L) by the PHA content (% dry weight of the cells)

<sup>d</sup>Averages do not include oleic acid values

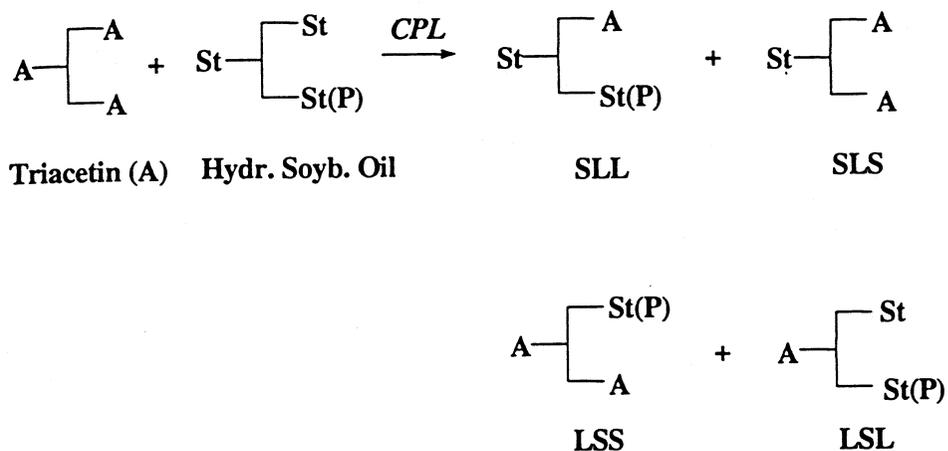
**TABLE 2  $\beta$ -Hydroxy fatty acid repeat unit composition of poly(hydroxyalkanoate) polymers produced by *P. resinovorans* grown on triglyceride substrates**

	$\beta$ -hydroxymethyl ester (%) <sup>a</sup>									
	C <sub>4:0</sub>	C <sub>6:0</sub>	C <sub>8:0</sub>	C <sub>10:0</sub>	C <sub>12:0</sub>	C <sub>12:1</sub>	C <sub>14:0</sub>	C <sub>14:1</sub>	C <sub>14:2</sub>	C <sub>14:3</sub>
PHA-T	Tr	3	15	46	17	4	6	9		
PHA-L	Tr	7	26	34	14	4	4	8	3	
PHA-B	Tr	9	31	35	15	Tr	4	5		
PHA-O	1	8	29	33	14	1	3	10	1	
PHA-Su	2	5	22	35	14	3	3	13	3	
PHA-So	Tr	4	18	32	8	14	4	9	10	Tr
PHA-C	Tr	7	33	40	16	1	3	Tr		

<sup>a</sup>Data are gas chromatographic (GC) area percents of methyl esters of the  $\beta$ -hydroxy acids produced by the acid hydrolysis of the PHA polymers found in Table 1.

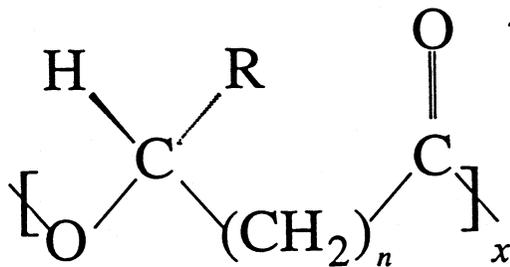
## SCHEME 1

### Synthesis of Low-Calorie Triglycerides



## SCHEME 2

### General Structure for Poly( $\beta$ -hydroxyalkanoate) Polymers



- 1) Short-chain length PHA (sci-PHA) where  $n = 1$  and  $R = 1, 2, \text{ or } 3$  carbon atoms
  - a) Poly(3-hydroxybutyrate) (PHB)
  - b) Poly (3-HB-co-3-hydroxyvalerate) (PHB/V)
- 2) Medium chain length-PHA (mcl-PHA) where  $n = 1$  and  $R = 4\text{-}11$  carbon atoms

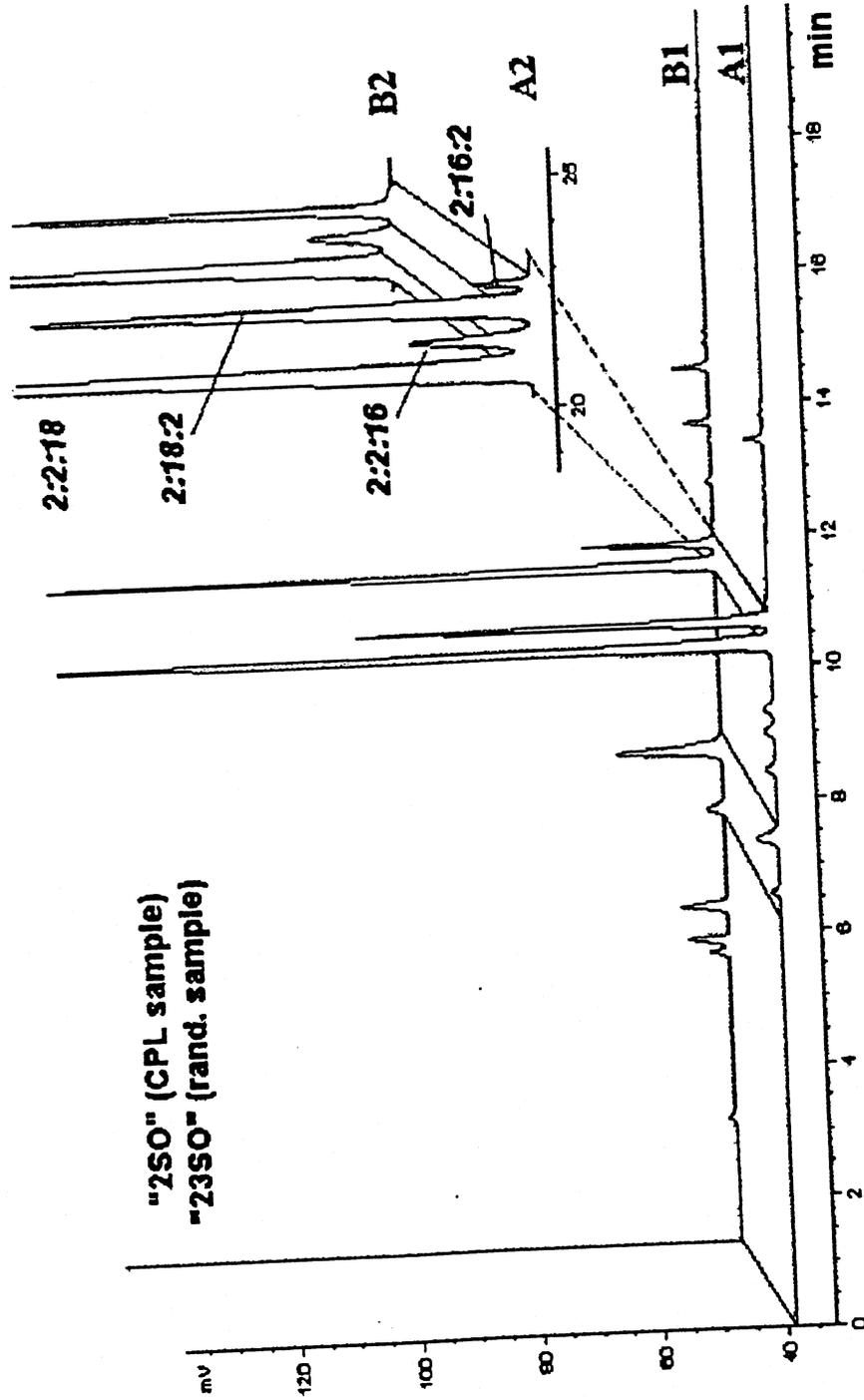


Figure 1. High performance liquid chromatographic (HPLC) separation of the short and long chain triacylglycerol (SLCT) products from the chemical (A1 and 2) and the CPL-catalyzed (B1 and B2) interesterification of hydrogenated soybean oil (HSO) with triacetin. Chromatograms A1 and B1 obtained with a cyanopropyl column and B1 and B2 obtained with a silica column.

# Oxygenation of Linoleic Acid

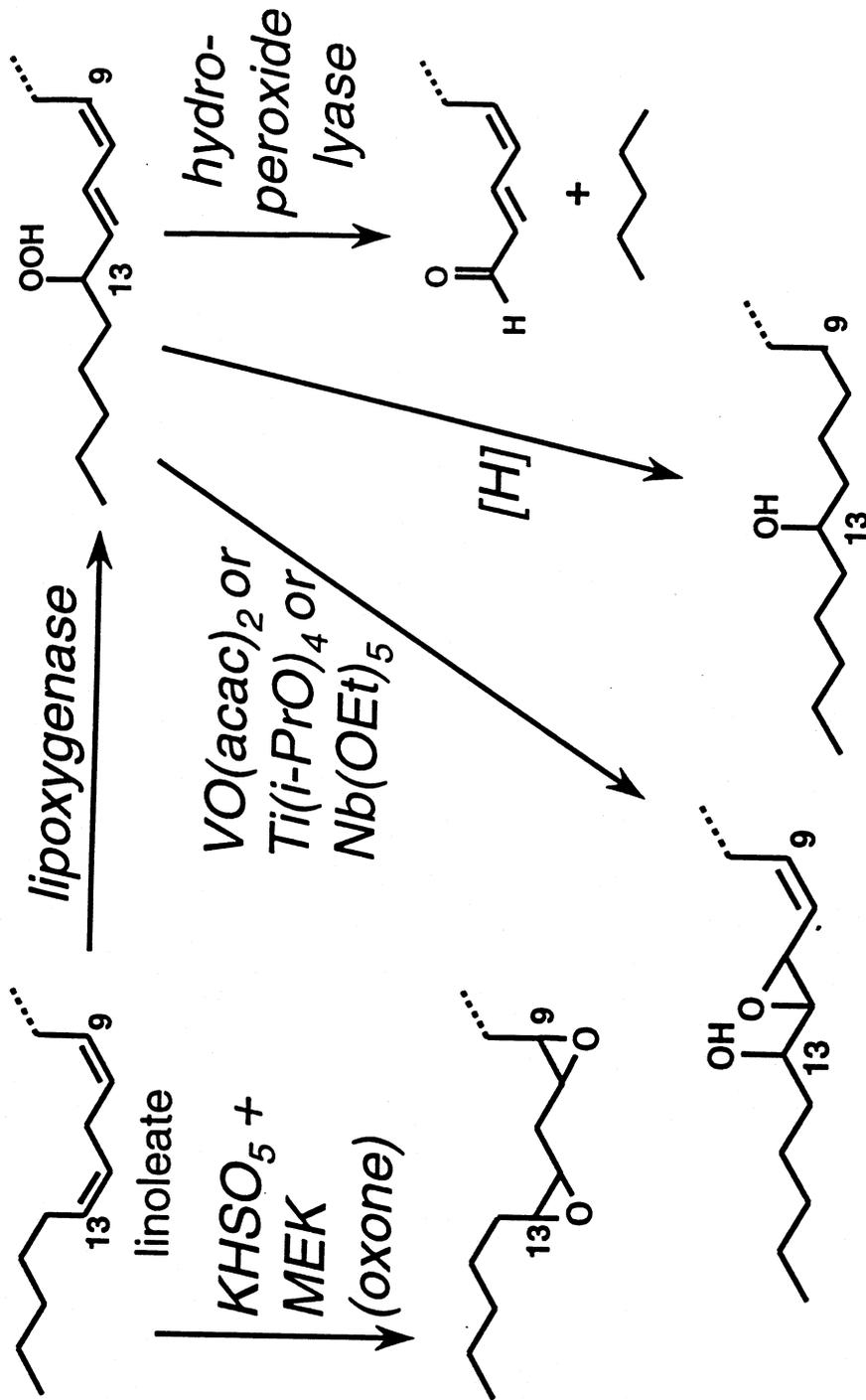


Figure 2. Conversion of polyunsaturated fatty acids to oxygenated fatty acid derivatives. The left side shows epoxidation of linoleate using the oxone method. The right side shows lipoxygenase conversion of linoleate to its 13-hydroperoxy derivative, which is then converted to the corresponding alcohols, aldehydes, and epoxy hydroxy compounds.

### Prospective Uses for Oxo-Acid from HPLS Cleavage of Fatty Acid Hydroperoxides

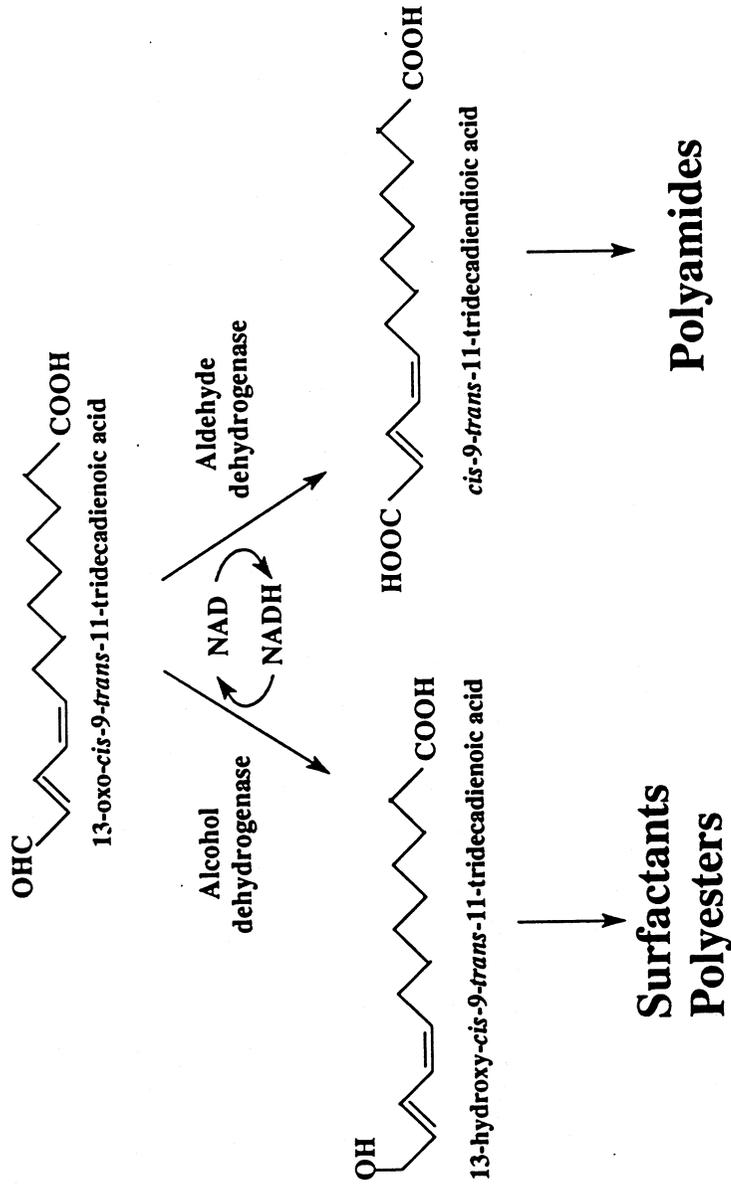
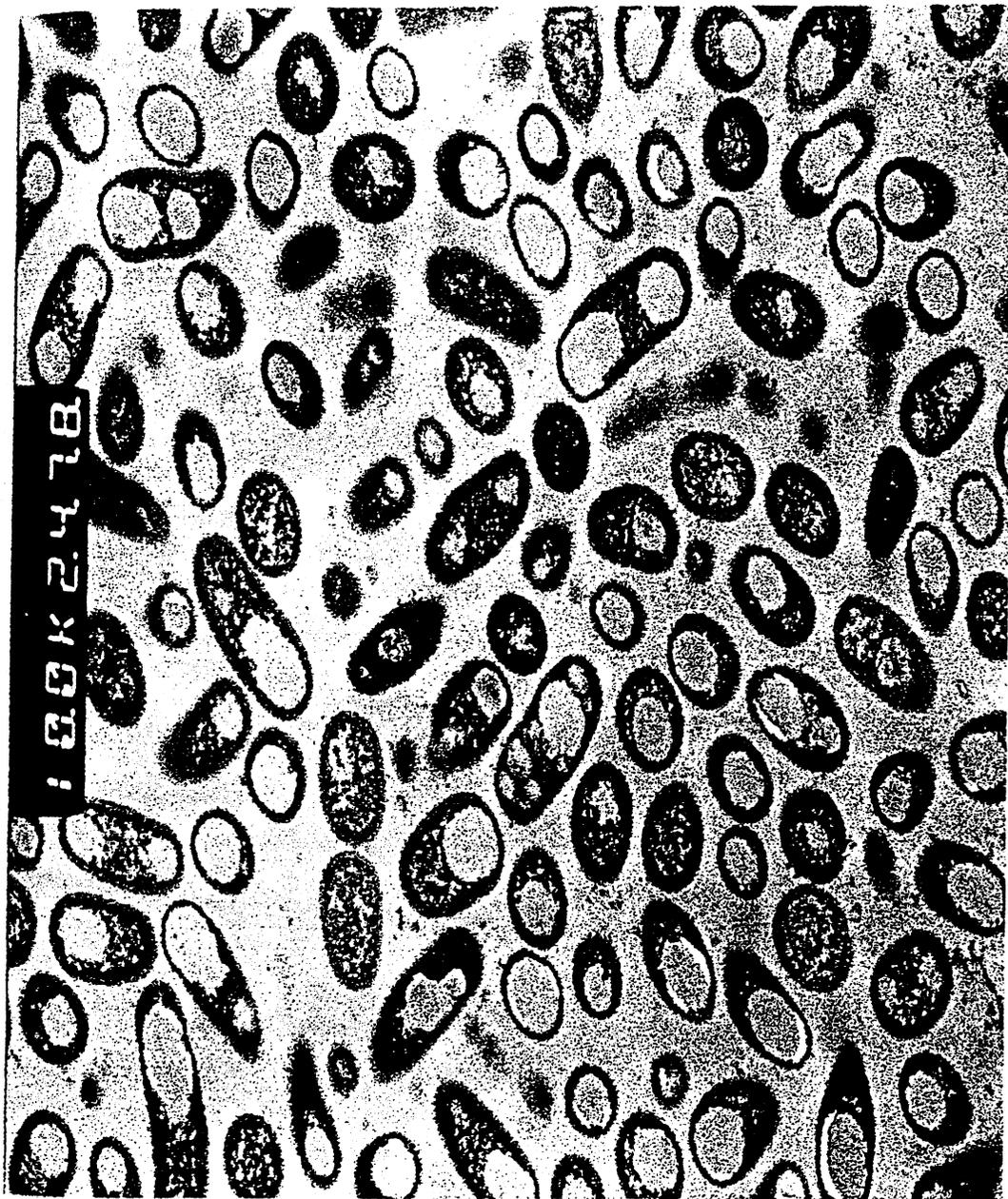


Figure 3. Oxidative cleavage of 13-hydroperoxy linoleic acid (HPODE) catalyzed by the enzyme hydroperoxide lyase (HPLS).



*Ps. saccharophila* NRRL B-628 (Exp. DKS-I-89)

Figure 4. Thin-section electron microscopic images of *Ps. saccharophila* NRRL B-628 grown in a chemically defined medium containing tallow as the sole carbon source.