Biotechnology and Oleochemicals: Changing Patterns

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ABSTRACT

Biotechnology will have a broad impact on the oleochemicals industry. Only a narrow range of this interface is discussed: (a) the use of organic solvents in the enzymatic synthesis of lipid derivatives, (b) the effect of the chemical nature of the feedstock on the production of microbial monoesters, and (c) temperature as a determinant of the level of unsaturation in biosynthetic lipids. Interest in running enzymatic reactions in high concentrations of organic solvents is increasing. The implications of such processes for the oleochemical industry is illustrated by examples of ester synthesis and interesterification of oils and fats. The dramatic effect of feedstock chemistry on the final monoester product mix produced by *Acinetobacter* sp. HO1-N is also discussed. Products resulting from using *n*-alkanes (C_{16}-C_{20}), acetic and propionic acids and, most recently, ethanol and propanol, are illustrated. They range from a monoester mix resembling sperm oil to one similar to jojoba oil. In general, temperature inversely affects biolipid unsaturation: the lower the temperature, the greater the unsaturation. The major function of this response is to preserve fluidity and function in biological membranes. The effect is universal in nature, occurring in animals, plants and microorganisms. Controlled laboratory studies have supported these observations made in nature. We have investigated the effect of temperature on the unsaturation of monoesters produced by the bacterium, *Acinetobacter* sp. HO1-N. The inverse relationship between temperature and unsaturation is clearly shown. The enzymatic basis for these results and the possibility of chemical or genetic modification of plants and microorganisms to produce more or less unsaturated lipids is briefly discussed. Organic solvents, feedstock chemistry and temperature stress in biocatalysis are but three of the variables at the interface of biotechnology and the oleochemicals industry that will cause changing patterns.

INTRODUCTION

The purpose of this paper is to illustrate with three examples how biotechnology can affect present patterns in oleochemicals. The three areas to be discussed are: (a) the use of enzyme reactions in the presence of high levels of organic solvents to synthesize useful oleochemicals, (b) the dramatic effect of feedstock chemistry on monoester production by *Acinetobacter* sp. HO1-N, and (c) the relationship between temperature and unsaturation of monoester mixtures synthesized by this bacterium. A broader view of the potential for biotechnology to alter the oleochemical field can be obtained by reading some of the fine reviews that have been published recently (1-5).

ENZYME REACTIONS IN ORGANIC SOLVENTS

Despite the natural occurrence of enzyme reactions in lipophilic environments, e.g., in cellular membranes, it is generally assumed that organic solvents are unfavorable to enzyme activity, certainly to commercial applications of biocatalysis. Often, solvents do inhibit enzyme activity but, with increasing frequency, it is being demonstrated that there is a positive side to biocatalysis in organic solvents.

There are several mechanisms by which this positive effect may be achieved: increase in reactant solubility, limiting the concentration of water, and alteration of active-site conformation in enzymes.

Both biphasic and monophasic systems have been studied. In biphasic systems with an aqueous and a water-immiscible solvent phase, there is an advantage in that the enzyme in the aqueous phase is not exposed to high solvent concentrations. In cases of enzymatic transformation of substrates such as steroids that are poorly soluble in water, a major improvement in percentage conversion may be obtained by dissolving the substrate in a suitable solvent. In Table I, the percentage conversion of cortisone to preg-4-en-17α, 20β, 21-triol-3,11-dione is a result of balancing steroid solubility in the solvent and solvent solubility in water. Although the best solvents, butyl and ethyl acetate, inhibit 20β-hydroxysteroid dehydrogenase activity, they compensate by offering a favorable balance of substrate and solvent solubility (6). Another application of the biphasic approach with commercial potential is in cases where the synthesis of compounds, such as esters, is thermodynamically unfavored in water. The level of organic solvent in the system can be up to 99%, with water as a limiting reagent, thus reducing hydrolysis. An example of such a reaction is illustrated in Table II(A) with chymotrypsin wherein the ester is synthesized in the presence of chloroform rather than water as the major solvent (7).

In Table II(B) are shown a number of examples of monophasic systems for ester synthesis using dried mycelia of *R. arrhizus*. In these cases, the solvent is the alcohol reactant (8-10). Techniques such as these could synthesize novel esters for use as flavors and fragrances.

Table III indicates that these methods can produce mono- and diglycerides also. By dissolving glycerol and fatty acid in solvent, then contacting this mixture with dried mycelia of *R. arrhizus*, good yields of glycerides were obtained in flask or reactor configurations. Such a tech-

TABLE I

Improvement by Organic Solvents of Cortisone Reduction by 20β-Hydroxysteroid Dehydrogenase (20β-HSDH)

Organic solvent	20β-HSDH activity (% inhibition)	Cortisone solubility (g/100 mL)	Organic solvent/water solubility (%, w/v)	Cortisone conversion (%)
n-Hexane	0	0.002	0.014	<5
Carbon tetrachloride	0	0.004	0.08	<5
Chlorobenzene	0	0.030	0.048	15
Diethyl ether	62	0.017	7.5	10
Butyl acetate	52	0.160	0.5	100
Ethyl acetate	71	0.270	8.6	90

TABLE II

Ester Synthesis by "Hydrolytic" Enzymes in Solvents

(A)		
N-acetyl L-tryptophan + ethanol	chymotrypsin / $CHCl_3$	N-acetyl L-tryptophan ethyl ester (100%; in H_2O, 0.01%)
(B)		
linoleic acid + cetyl alcohol		cetyl linoleate (∼90%)
palmitic acid + octyl alcohol		octyl palmitate (∼90%)
pentanoic acid + pentyl alcohol	dried mycelia of *Rhizopus arrhizus*	pentyl pentanoate
butyric acid + benzyl alcohol		benzyl butyrate
acetic acid + geranyl alcohol		geranyl acetate (30-70%)

TABLE III

Glyceride Synthesis in Solvent by Dried *Rhizopus arrhizus* Mycelia

Shake flask:

glycerol (0.2% w/v) + oleic acid (10% w/v)	*R. arrhizus* / acetone	1-monoglyceride 1,2/1,3-diglycerides 1,2,3-triglyceride (trace)

70% yield based on conversion of hydroxyl groups to ester.

Packed bed or stirred tank reactors:

glycerol (0.2% w/v) + linoleic acid (10% w/v)	*R. arrhizus* / diisopropyl ether (4) acetone (1) (<0.05% H_2O, 1 mL min^{-1})	1-monoglyceride 1,3-diglyceride 1,2,3-triglyceride (trace)

45% yield based on conversion of hydroxyl groups to ester.

nique could produce tailor-made glycerides for special applications. A similar process with *Corynebacterium* sp. 5-401 in *n*-hexane converted glycerol and oleic acid to triolein, but not to mono- or diolein (11).

As in Table I with cortisone reduction, the nature of the solvent employed for ester synthesis is important in the design of an efficient process. Table IV shows that chloroform was the best solvent in a system using chymotrypsin (12).

Interesterification is another example of enzyme reactions in biphasic systems. The general objective is to change the fatty acid components of a triglyceride or mixture of triglycerides by exchange with either free fatty acids or the fatty acids of other triglycerides. By such a process, a cheap oil such as olive oil can be converted into an approximation of the more commercially valuable cocoa butter. A typical reaction system for interesterification employs lipase-coated inorganic particles, activated with 10% water, stirred in a tank with reactants dissolved in a suitable solvent such as petroleum ether (13).

Chemically catalyzed interesterification using sodium or sodium alkoxide results in random acyl migration and exchange. Although random interesterification can also be obtained with nonspecific lipases (Table V), the existence of 1,3-specific and fatty acid-specific lipases allows for the synthesis of new products not available via random reactions.

TABLE IV

Effect of Solvents on the Synthesis of N-Benzoyl-L-Phenylalanine Ethyl Ester by α-Chymotrypsin

Solvent	% Yield
Chloroform	80
Benzene	64
Carbon Tetrachloride	63
Diethyl Ether	26
Water	0

TABLE V

Interesterification of Coconut and Olive Oils (1:1, w/w)

Triglyceride fatty acids carbon no.	Starting mixture wt %	Interesterified oils % change from starting mixture in interesterified oils treated with	
		Alkali metal	*C. cylindracae* lipase
26-38	29.2	−14.0	−13.0
40-48	12.0	+48.9	+48.3
50-56	58.7	−34.8	−35.2

TABLE VI

Triglyceride Interesterification of Olive Oil and Stearic Acid (5:1, w/w) with a 1,3-Specific Lipase of *Rhizopus delemar*

	Total triglycerides		2-Position		1- and 3- Positions	
Fatty acid	wt % in olive oil	Interesterified oil % change	wt % in olive oil	Interesterified oil % change	wt % in olive oil	Interesterified oil % change
16.0	16.6	−2.9	3.5	−0.3	23.2	−4.3
16:1	1.8	−0.2	1.3	+0.3	2.0	−0.4
18:0	2.0	+13.6	1.0	−0.3	2.5	+20.5
18:1	66.8	−10.2	72.0	+0.2	64.2	−15.4
18.2	12.8	−0.2	22.2	+0.1	8.1	−0.4

Table VI illustrates the interesterification of olive oil and stearic acid using a 1,3-specific lipase, wherein the 1,3-positions of olive oil are enriched in stearic acid.

Table VII shows that in the presence of a linoleic-specific lipase, olive oil is enriched in linoleic acid, not stearic acid.

These data (13) indicate that the ratio and positions of saturated and unsaturated fatty acids can be altered by using specific enzymes and suitable substrates.

A final demonstration of the effects of solvents on enzymes involves a modification of enzyme specificity by a solvent-mediated change in active-site conformation. Table VIII illustrates the effect of dimethylsulfoxide (DMSO) on α-thrombin, an enzyme with both esterase and amidase activity. DMSO increases esterase activity and decreases amidase activity (14).

Thus, it is clear that the mechanisms by which solvents can advantageously influence an enzyme reaction are multiple and complex. The development of commercial biocatalytic processes involving high-solvent conditions is only beginning.

FEEDSTOCK VARIATION

By feedstock variation, the nature of the monoester (wax ester) products obtained from a biological system can be altered within limits set by the biochemical machinery of the organism, in this paper *Acinetobacter* sp. HO1-N.

Previous reports of the production of monoesters by HO1-N from *n*-alkanes are shown in Table IX. The number of carbon atoms in the monoesters varied in relation to the number of carbon atoms in the *n*-alkane substrate. Of particular importance is the fact that all reported monoesters were saturated (15). Packed column gas chromatography was used to analyze the monoester composition in these studies. When capillary gas chromatography was used instead, it was discovered that HO1-N actually metabolized *n*-alkanes, as well as acetic acid and ethanol, to mixtures of monoesters containing 0, 1 or 2 carbon-carbon double bonds (at ω7 and ω9; Table X). Figure 1 shows the relative positions in a gas chromatograph of saturated, monoenic and dienic wax esters produced from *n*-hexadecane.

Figure 2 shows the comparative composition of the monoester mixtures obtained by the metabolism of *n*-hexadecane (C-16), *n*-eicosane (C-20) and acetic acid. Each substrate gives a distinctive monoester product.

Figure 3 illustrates the wax esters produced from ethanol and propanol. As with acetic acid, the monoesters from ethanol are primarily even in carbon number. With propionic acid (and the 1:1 mixture of acetic and propionic acids), a substantial amount of odd carbon-number monoesters is synthesized. Figure 4 shows a similar, but more dramatic case with acetic acid and propionic acid.

TABLE VII

Interesterification Using the Linoleic Acid Specific Lipase of *Geotrichum candidum* with Olive Oil Triglyceride, Stearic Acid and Linoleic Acid (1.0:0.15:0.15, w/w/w)

Fatty acid	Olive oil (%)	Interesterified oil (% change)
16:0	11.6	−0.1
16:1	0.8	−0.3
18:0	3.6	+0.9
18:1	72.8	−8.0
18:2	10.6	+7.7
20:1	0.6	−0.2

TABLE VIII

Effects of DMSO on the Enzyme Activity of Bovine α-Thrombin

	Activity (%)	
DMSO (% v/v)	Esterase[a]	Amidase[b]
0	100	100
5	135	60
10	175	30
15	190	30
20	200	9

[a]1 mM *p*-Tosyl-L-arginine methyl ester · HCl.
[b]0.2 mM N-Benzoyl-L-phenylalanyl-L-valyl-arginine-*p*-nitroanilide · HCl.

TABLE IX

Previous Reports of Wax Esters Produced by *Acinetobacter* sp. HO1-N from *n*-Alkanes

n-Alkane	Saturated wax esters
n-Tetradecane (C_{14})	$CH_3(CH_2)_{12}CO_2(CH_2)_{13}CH_3$
n-Hexadecane (C_{16})	$CH_3(CH_2)_{14}CO_2(CH_2)_{15}CH_3$
n-Heptadecane (C_{17})	8:1:1 mixture of $CH_3(CH_2)_{15}CO_2(CH_2)_{16}CH_3$ $CH_3(CH_2)_{14}CO_2(CH_2)_{16}CH_3$ $CH_3(CH_2)_{13}CO_2(CH_2)_{16}CH_3$
n-Octadecane (C_{18})	1:1 mixture of $CH_3(CH_2)_{16}CO_2(CH_2)_{17}CH_3$ $CH_3(CH_2)_{14}CO_2(CH_2)_{17}CH_3$

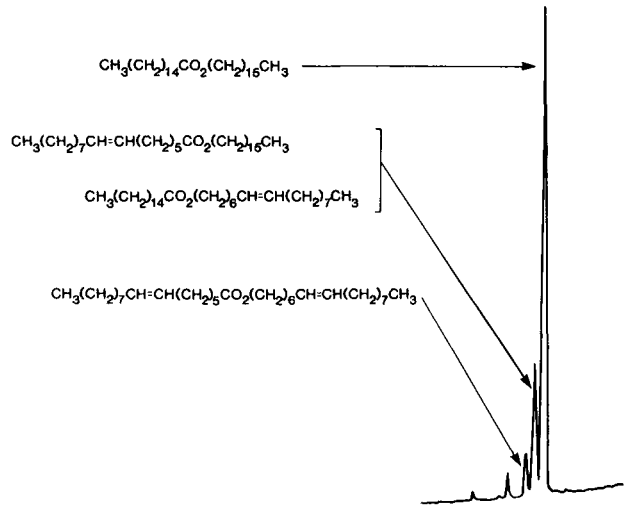

FIG. 1. Capillary GC-MS chromatogram illustrating the relative positions of monoesters produced by *Acinetobacter* sp. HO1-N from *n*-hexadecane as a function of unsaturation.

TABLE X

Formation of Wax Esters by *Acinetobacter* sp. HO1-N from Various Substrates

Substrate ⟶	Fatty acids +	Fatty alcohols ⟶	Wax esters
n-Hexadecane	16:0, 16:1[a]	16:0, 16:1	32:0 32:1 32:2
n-Eicosane	16:0, 16:1 18:0, 18:1 20:0, 20:1	20:0, 20:1	36:0 38:0 40:0 36:1 38:1 40:1 36:2 38:2 40:2
Ethanol and acetic acid	16:0, 16:1 18:0, 18:1	16:0, 16:1 18:0, 18:1	32:0 34:0 36:0 32:1 34:1 36:1 32:2 34:2 36:2

[a]16, 18, 20, 32, 34, 36, 38, 40 = number of carbon atoms.
0, 1, 2 = number of double bonds.

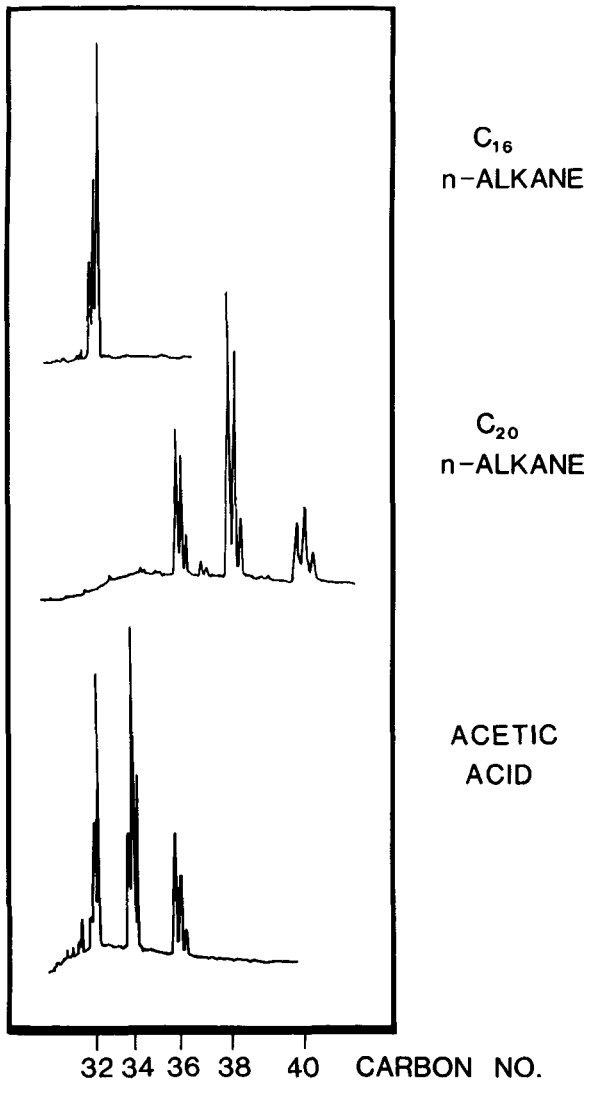

FIG. 2. Capillary GC-MS chromatogram illustrating monoester mixtures produced by *Acinetobacter* sp. HO1-N from various feedstocks.

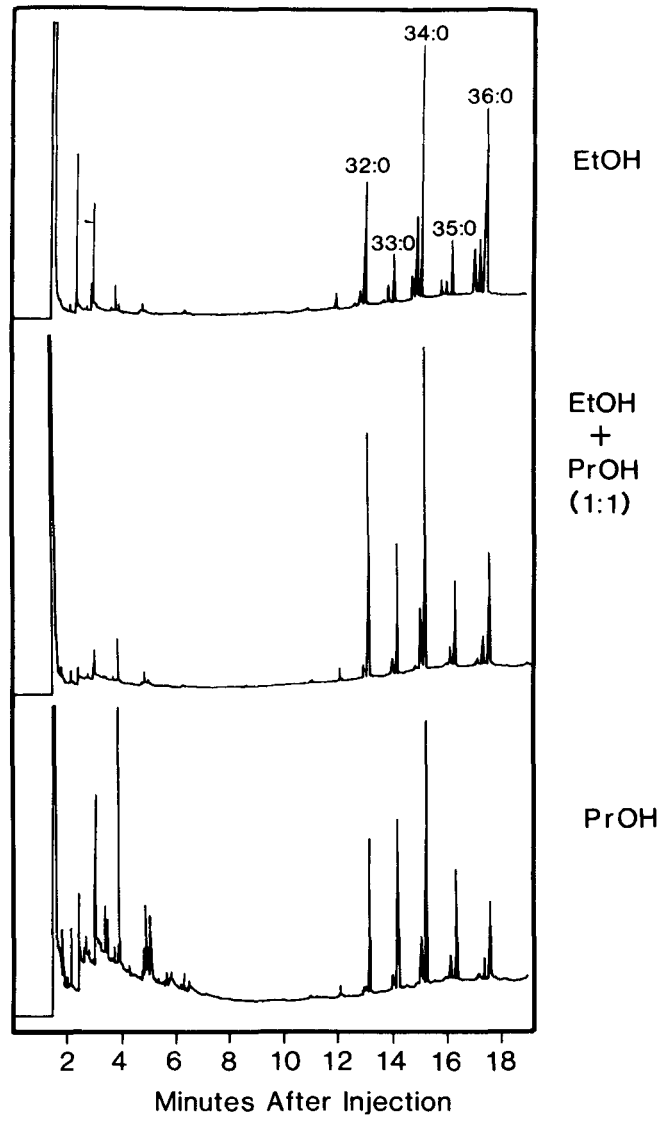

FIG. 3. Capillary GC-MS chromatogram illustrating monoester mixtures produced by *Acinetobacter* sp. HO1-N from ethanol, propanol and ethanol/propanol (1:1, v/v).

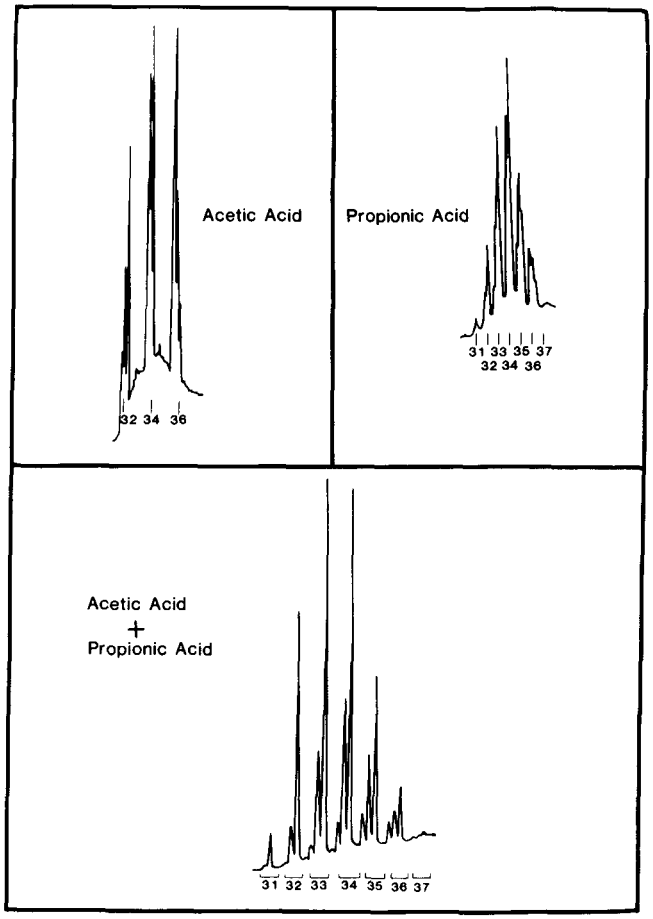

FIG. 4. Capillary GC-MS chromatogram illustrating monoester mixtures produced by *Acinetobacter* sp. HO1-N from acetic acid, propionic acid and acetic acid/propionic acid (1:1, w/w).

Table XI summarizes the versatility of HO1-N in producing a range of wax ester mixtures whose composition depends on the feedstock chemistry. These wax ester mixtures mimic those produced by other living organisms such as the jojoba plant and the sperm whale.

The development of an economic and commercially significant process depends on optimization of wax ester production to give maximum percentage conversion of substrate-to-product and the identification of a product having sufficient value to warrant such development.

TEMPERATURE AND MONOESTER UNSATURATION

The versatility of HO1-N in producing various wax ester mixtures as a function of feedstock chemistry can be further expanded by imposing a temperature stress on the biosynthetic system.

In general, but not without exception, an inverse relationship exists in nature between temperature and unsaturation in responsive lipid classes: low temperature, high unsaturation; high temperature, low unsaturation. The major function of this biochemical response is to maintain membrane fluidity and function. The reaction may be mediated by temperature-sensitive desaturases, although it has also been suggested that oxygen availability influenced by temperature is a major determinant (16,17), a conclusion denied by others (18,19).

To review in respectable detail the literature pertinent to the temperature-lipid unsaturation relationship is beyond the scope of this paper. Relevant reviews should be consulted, although none are satisfyingly complete (1,18, 20-23). In microorganisms, plants and animals, the fatty acids most commonly reported to be increased by low temperatures are linolenic (18:3), linoleic (18:2) and oleic (18:1) acids at the expense of the appropriate, more saturated palmitic (16:0), oleic or linoleic acid (17,24-47).

Wax ester biosynthesis by *Acinetobacter* sp. HO1-N from various substrates clearly shows the inverse relationship between temperature and unsaturation. Table X indicates the nature of the wax ester mixtures obtained from certain *n*-alkanes, acetic acid and ethanol. The wax esters contain 0, 1 or 2 double bonds. In the case of a monoenic wax ester, the unsaturation may be in either the acyl or alkoxy moiety of the ester. In the case of a dienic wax ester, one double bond is in the acyl moiety and the other is in the alkoxy moiety (15,48-51).

Figure 5 and the data in Table XII illustrate the effect of temperature on the unsaturation of wax esters from *n*-hexadecane. As the growth temperature is lowered from 30 C to 24 C to 17 C, the level of unsaturation in the wax esters increases (49,50). Figure 6 and Tables XIII and XIV show even more impressive effects when HO1-N is grown on *n*-eicosane or ethanol (49-51).

Calculation of a wax ester unsaturation index as shown in Table XV indicates that decreasing temperature increases the index and that, for any given temperature, the index for *n*-eicosane > ethanol > *n*-hexadecane. These data clearly implicate temperature as a determinant in wax ester

TABLE XI

Distribution of Major Wax Esters from Various Biological Sources

	Number of C atoms in wax esters										
Source	28	30	32	33	34	35	36	38	40	42	44
Orange roughy					+		+	M[a]	+	+	
Sperm whale	+	+	M		+		+				
Jojoba bean								+	+	M	+
Acinetobacter HO1-N											
n-hexadecane			M								
n-octadecane					+		M				
n-eicosane							+	M	+		
n-docosane									+	M	+
acetic acid			+		M		+				
ethanol			+		M		+				
propionic acid			+	+	M	+	+				
propanol			+	+	M	+	+				

[a]M = main ester.

TABLE XII

Temperature Effect on Unsaturation of Wax Esters Produced from N-Hexadecane by *Acinetobacter* sp. HO1-N

Wax esters	% Composition		
	17 C (48 hr)	24 C (24 hr)	30 C (48 hr)
32:0	50	61	80
32:1	35	31	18
32:2	15	8	2

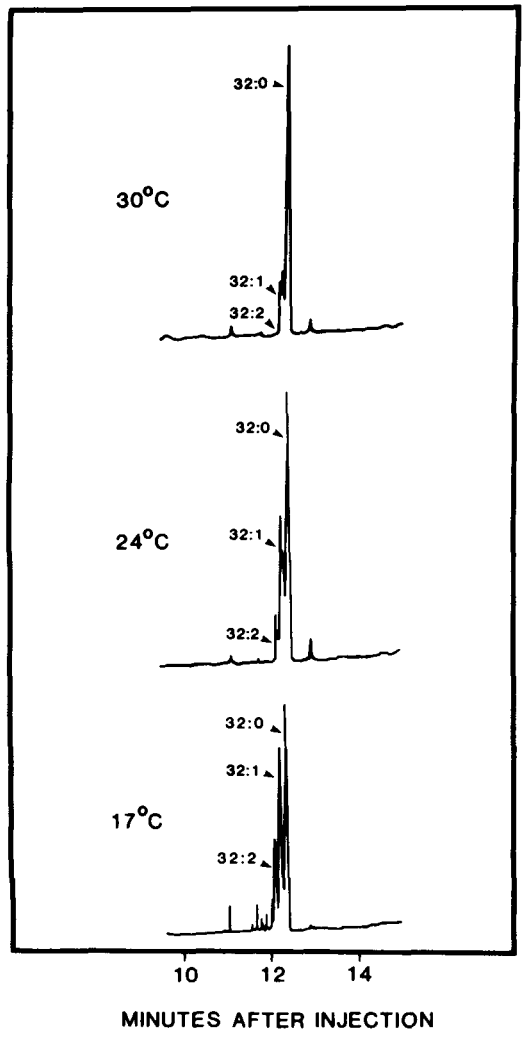

FIG. 5. Capillary GC-MS chromatogram illustrating the effect of temperature on the unsaturation of monoesters produced by *Acinetobacter* sp. HO1-N from *n*-hexadecane.

unsaturation. Whether the relationship is a direct one between temperature and temperature-responsive biocatalysis, or is an indirect one initiated by temperature but mediated by a second and direct determinant such as oxygen, cannot be judged at present—and for practical purposes, may be only academic since temperature may be a more readily controllable effector.

TABLE XIII

Temperature Effect on Unsaturation of Wax Esters Produced from N-Eicosane by *Acinetobacter* sp. HO1-N

Wax esters		% Composition		
		17 C (48 hr)	24 C (24 hr)	30 C (48 hr)
36:0		2	7	8
38:0		1	8	9
40:0		2	7	8
	Total	5	22	25
36:1		8	14	16
38:1		10	15	18
40:1		6	8	6
	Total	24	37	40
36:2		26	16	13
38:2		33	21	19
40:2		12	4	3
	Total	71	41	35

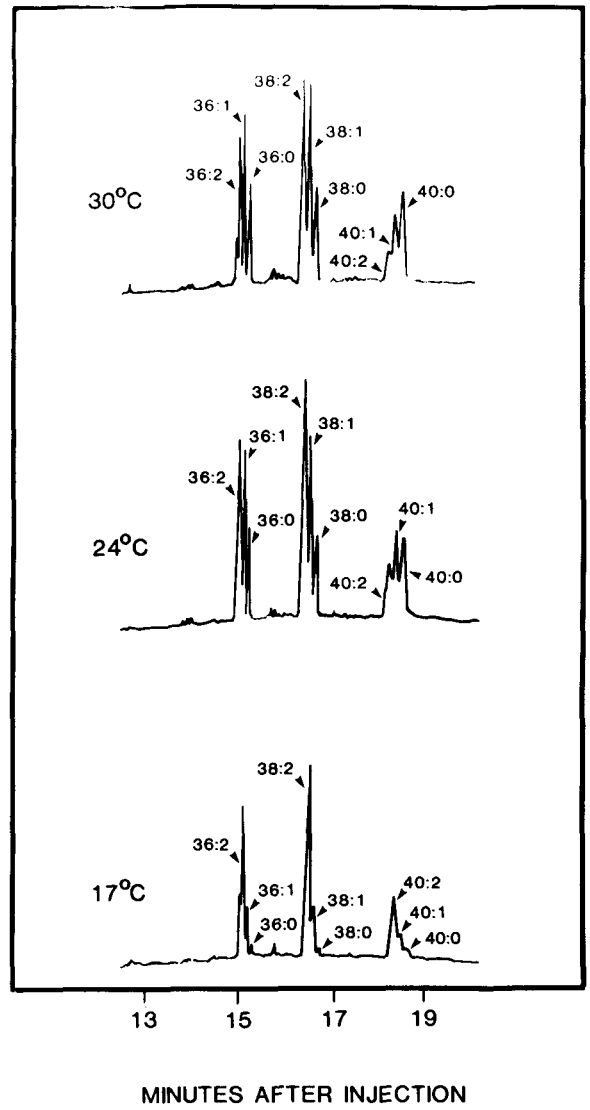

FIG. 6. Capillary GC-MS chromatogram illustrating the effect of temperature on the unsaturation of monoesters produced by *Acinetobacter* sp. HO1-N from *n*-eicosane.

TABLE XIV

Temperature Effect on Unsaturation of Wax Esters Produced from Ethanol by *Acinetobacter* sp. HO1-N

Temperature (C)	Diene fractions (32:2, 34:2, 36:2) Total (%)	Monoene fractions (32:1, 34:1, 36:1) Total (%)	Saturated fractions (32:0, 34:0, 36:0) Total (%)
17	72	18	10
24	29	40	31
30	9	25	66

TABLE XV

Effects of Substrates and Temperature on Unsaturation Indices of Wax Esters Produced by *Acinetobacter* sp. HO1-N

Substrate	Temperature (C)	Unsaturation index[a]
n-Hexadecane	17	0.65
	24	0.47
	30	0.22
Ethanol	17	1.62
	24	0.76
	30	0.43
n-Eicosane	17	1.66
	24	1.19
	30	1.10

[a](% Monounsaturated + 2X % diunsaturated)/100).

OBSERVATIONS

Even in this brief discussion of the effects of three variables (solvents, feedstocks and temperature) on oleochemical biosynthesis, it is evident that novel products, processes and markets will be created by biotechnology in the near future.

To expand the vision slightly, mention should be made of contributions to be made by chemicals and genetic engineering in modifying oleochemical production by biological systems. It has been known for some time that ethanol and chaotropic chemicals such as trichloroacetic acid can cause an increase in membrane lipid unsaturation in *Escherichia coli* (52). Recent work has shown that pyridazinone derivatives can regulate levels of linoleic or linolenic acids in responsive plant material, and affect the ability of these plant materials to survive low or elevated temperatures (25,53, 54). Such chemicals could, in conjunction with natural temperature stresses, result in new methods for weed control and an expansion of food crop cultivation and oleochemical production into normally hostile climates. This, of course, depends on the cost of applying such compounds in the field.

The most powerful tool in altering oleochemical production by biological systems is genetic engineering. It is inevitable that genetic constructs will be created that will provide almost any desired chemical composition, within the limits of the biochemical competency of the living material. A clear example is the instance of two varieties of safflower, US-10 and UC-1, in which the former yields mainly linoleic acid and the latter linolenic acid in the mature seed (55).

This short diversion is merely to illustrate further that as our sophistication in discovering and applying new principles of biotechnology in the field of oleochemicals grows, unexpected, novel and profitable processes will be designed and will become commercially important.

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Current Pollution Control Practices in the United States

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ABSTRACT

An overview of pollution control practices in the United States is presented here, with particular emphasis on those factors which motivate and direct current practices. A more in-depth discussion is provided of certain areas, particularly those items which might be unique to the USA. More emphasis has been placed on wastewater-related issues. The largest dollar expenditures and technical efforts have probably gone to solving these problems in the oleochemical field. Some example projects are presented to demonstrate the significant points.

OVERVIEW OF FACTORS AFFECTING POLLUTION CONTROL DECISION-MAKING

The keynote of this paper is the need to address environmental problems in the oleochemical industry in the USA from a standpoint which considers all factors. Resolution of one problem without considering its overall implication usually serves only to create another problem.

Implementation of current pollution control practices is significantly affected by a variety of factors. These will generally come under one of four categories: (a) government regulatory control; (b) in-plant control and process modification; (c) available and developing technology for wastewater, or stack emission treatment; and (d) interaction of air, water and solid waste problems.

Although this all seems simple enough, those who work in the field on a routine basis recognize that this is often like an air-inflated balloon bulging on one side—as one pushes in the bulge, another often forms on the other side. Decisions cannot be (and usually are not) made without consideration of all factors as well as the details and cost implication associated with each alternative solution. Often one consideration receives compromise at the expense of another.

For example, a decision as to whether to build a waste-treatment facility at an existing processing plant is usually initially motivated by government direction or regulation. The further decision as to what type of treatment plant should be built is often predicated on what can be done with solid sludges, and what reduction to waste load can be made through process control.

In some very rare instances, one might choose, as an alternative, to close one's plant entirely or, more realistically, to eliminate a certain product line.

GOVERNMENT ENVIRONMENTAL REGULATORY STRUCTURE

Environmental matters are regulated everywhere in the USA on both federal and state levels and often also by local municipal and/or county ordinances.

The Environmental Protection Agency (EPA)—the Federal Agency—is divided into 10 regions throughout the country. The EPA generally issues guidelines and regulations for industrial and municipal environmental enforcement, and overviews similar state programs dealing directly with generators of air, water and solid wastes. In practice, then, industry must deal with regulations issued by both federal and state governments. If a local government is involved, it usually also has its own regulations, particularly with respect to discharge of pretreated industrial wastes to sanitary sewage systems. A much smaller number of municipalities have their own active air pollution control and hazardous waste administrations. Nonhazardous solid wastes are almost universally dealt with on the local level.

This is conducive to confusing and often conflicting sets of regulations concerning a given problem with which industry must deal. Regulations and, more importantly, governmental enforcement postures vary greatly. The confusion is extended further for larger companies in that programs, regulations and enforcement vary significantly from state to state and even one federal EPA region to another, thus causing similar plants to be faced with dissimilar requirements.

The environmental regulatory programs have been in place for ca. 15 years. As a result, some of the state and