Further Aspects of Wax Ester Biosynthesis by *Acinetobacter* sp. HO1-n

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ABSTRACT

Earlier reports from our laboratory have shown that *Acinetobacter* sp. HO1-N can synthesize wax esters from diverse substrates: individual n-alkanes, acetic acid, propionic acid, ethanol and propanol. The chemical nature of the wax ester mixture obtained is both substrate and temperature dependent. In a continuation of these studies, we have found that the use of n-alkane mixtures, including primary gas oil, yields an even broader range of wax ester mixtures than previously produced from single n-alkane substrates. In addition, two techniques for improving wax ester yields are discussed: the use of mutant strains of *Acinetobacter* sp HO1-N, and the presence of an excess of water-immiscible substrate, for example, n-hexadecane. In each case, it is speculated that a reduction of wax ester degradation contributes to the improved yields.

INTRODUCTION

Acinetobacter sp. HO1-N produces wax ester mixtures from a variety of substrates, including n-alkanes (1-8), n-alkenes (9), acetic and propionic acids (8), ethanol and propanol (8,10), and nutrient broth-yeast extract (3). These wax ester compositions can closely resemble sperm whale oil (C_{28}-C_{40} saturated, mono- and diunsaturated wax esters) and jojoba oil (C_{36}-C_{44} diunsaturated wax esters) and have commercial potential as replacements for "natural" wax ester mixtures. The compositional range of wax ester mixtures that can be synthesized by *Acinetobacter* sp. is wider than those of other sources since it can be varied experimentally by altering the nature of the substrate and the temperature. By so doing the average carbon chain length and level of unsaturation can be controlled (5-8). In this paper we report the ability to further broaden the wax ester composition obtained by using n-alkane mixtures rather than individual n-alkanes as substrates. In addition, we report that wax ester yields from *Acinetobacter* sp. HO1-N can be improved by the use of mutant strains and an excess of water-immiscible substrate, such as n-hexadecane.

EXPERIMENTAL PROCEDURES

Materials

All solvents used were of the highest purity available (Burdick and Jackson, Muskegon, MI, and MCB Reagents, Cincinnati, OH). N-alkanes were from Applied Science (State College, PA). Cetyl palmitate was from Aldrich Chemical (Milwaukee, WI). Wax ester standards were from Sigma Chemical (St. Louis, MO). N-alkane cut ($\sim C_{15}$-C_{30}) was from Standard Oil of Indiana (Tulsa, OK). Mueller-Hinton medium was obtained from Difco Laboratories (Detroit, MI).

Formation of Microbial Wax Esters

Several procedures for production of wax esters by *Acinetobacter* sp. HO1-N (ATCC 14987) that may be used (2,3) have been published. Inocula of parental and mutant strains for our studies of wax ester production from individual and mixed n-alkanes were prepared by growing the organisms on a mineral medium consisting of (in g/L): K_2HPO_4, 5; $(NH_4)_2HPO_4$, 10; $CaCl_2 \cdot 2H_20$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.04; KCl, 1; $MgSO_4$, 0.1; $MnSO_4 \cdot H_2O$, 0.04; Na_2SO_4, 0.05; NaCl, 0.02; Na acetate, 5; Na propionate, 5; pH 7.2. Cultures were grown in Erlenmeyer flasks (50 ml medium/250 ml size) on a rotary shaker (200 rpm) at 25 C for 18 hr.

Portions (25 ml) of the starter culture were used to inoculate Erlenmeyer flasks (1 L) containing 200 ml of modified mineral medium. The mineral medium was modified by replacing the sodium acetate and propionate with n-alkanes at 0.155 g/L of each n-alkane as needed. To facilitate dispersion in the medium, the n-alkanes were dissolved in the minimal amount of carbon disulfide and then added to the medium. The flasks were placed on a rotary shaker (250 rpm) at 25 C for 24 hr. Additional studies were made in which n-hexadecane was added in levels between 0.155-6.2 g/L.

In addition to studies with synthetic mixtures of n-alkanes, work was done with primary gas oil, a petroleum cut containing a range of n-alkanes ($\sim C_{15}$-C_{30}). The primary gas oil was added at 0.1% v/v with a 0.025% v/v supplement of n-hexadecane to facilitate ease of interpretation of the resulting wax ester mixture. The extra n-hexadecane favored the formation of C_{16}-fatty acid segments that were enzymatically coupled to variable alcohol segments formed from the primary gas oil n-alkane components.

Cell counts of inocula of parental and mutant strains were determined by spreading serial dilutions on agarized inoculum medium in petri dishes, three per dilution. The petri dishes were incubated at 25 C for 24-48 hr to obtain a consistent count.

Extraction and Analysis of Wax Esters

Wax esters were extracted from the fermentations with a 2:1 mixture of chloroform and methanol (2 x 50 ml portions/fermentation flask). The chloroform layer was separated and then concentrated at 80 C.

Wax esters were analyzed on a Hewlett-Packard 5880 A gas chromatograph equipped with a flame ionization detector. Sample dissolved in chloroform was injected (3 μl) into a fused-silica capillary column (30 m x 0.25 mm ID) coated with SE-54 (purchased from J & W Scientific, Rancho Cordova, CA), using a split injection mode (split ratio, 1:30). Helium flow through the column was set at 2 ml/min, and the column temperature was programmed from 200 C to 325 C at 10 C/min, then held at 325 C for 15 min. The injection port and the detector temperatures were set at 350 C. Wax ester standards were used for identification and quantitation of wax esters as previously described (5). Fused-silica capillary gas chromatography resolved the wax ester components according to their carbon chain length and their degree of carbon-carbon double bond unsaturation. The identity of each wax ester component was reported previously (5). In quantitating the wax esters and residual n-alkane substrates, a packed column (6 ft) containing 3% OV-1 was used with the same temperature programming as indicated for capillary columns.

Preparation and Isolation of Mutants

Acinetobacter sp. HO1-N was grown in Mueller Hinton broth for 24 hr at 25 C. The cells were collected, resuspended in 0.05 M Tris-maleic acid buffer pH 5 or 9 and then mutagenized with N-methyl-N'-nitro-N-nitro-

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soguanidine (MNNG, 200 μg/ml final concentration) for 1 hr at room temperature (11). After expression in Mueller Hinton broth, the mutagenized material was spread on petri dishes (100 x 15 mm) containing 25 ml of Mueller Hinton agar or agarized (1.5%) inoculum medium with 1% sodium acetate in place of 0.5% sodium acetate and sodium propionate. The plates were incubated for 18 hr at 25 C. Master plates for replicate plating were prepared by inoculating additional Mueller Hinton agar petri dishes with material from well-developed colonies on the Mueller Hinton and inoculum medium plates. The master plates were incubated for 18 hr at 25 C and were then used for replicate plating (12) on petri dishes containing agarized (1.5%) inoculum medium with either 1% sodium acetate or 4% cetyl palmitate as carbon source.

The petri dishes containing cetyl palmitate were prepared as follows:

To 250 ml double strength inoculum medium without carbon source, 10 g of cetyl palmitate, dissolved in 10 ml of carbon disulfide (CS_2), was added slowly. The solution was warmed very gently with stirring on a hot plate in a chemical hood until the CS_2 was eliminated. To the solution was added 250 ml of a 3% agar solution. The mixture was cooled with stirring to ~60 C and 4.5 ml of a filter-sterilized 1% solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) was added. Twenty-five ml of this medium was poured into each petri dish. The dishes were stored overnight at room temperature in a chemical hood without stacking prior to use.

The desired mutants were those that would grow on plates with 1% sodium acetate, but not on 4% cetyl palmitate. The TTC was present in the cetyl palmitate plates to aid in visualizing colonies, since growth was often poor. The sodium acetate plates were incubated at 25 C for 24 hr, and the cetyl palmitate plates were incubated for 96 hr.

The screen was designed to select mutants that would

TABLE I

Wax Esters Formed by *Acinetobacter* sp. HO1-N With Individual and Mixed n-Alkanes

n-Alkane(s) added	Number of C atoms in major wax esters produced						
	32	34	36	38	40	42	44
n-hexadecane (C_{16})	+						
n-octadecane (C_{18})		+	+				
n-eicosane (C_{20})			+	+	+		
n-docosane (C_{22})					+	+	+
$C_{16} + C_{20}$	+	+	+	+	+		
$C_{16} + C_{22}$	+	+	+	+	+	+	+
$C_{20} + C_{22}$			+	+	+	+	+
$C_{16} + C_{18} + C_{20}$	+	+	+	+	+		
$C_{18} + C_{20} + C_{22}$		+	+	+	+	+	+

TABLE II

Wax Esters Formed From n-Hexadecane and Primary Gas Oil by *Acinetobacter* sp. HO1-N

acyl segment $CH_3(CH_2)_{14}C(=O)-$	alkoxy segments $-O(CH_2)_{14\text{-}26}CH_3$	wax esters $CH_3(CH_2)_{14}C(=O)-O(CH_2)_{14\text{-}26}CH_3$
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{14}CH_3$	C_{31}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{15}CH_3$	C_{32}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{16}CH_3$	C_{33}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{17}CH_3$	C_{34}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{18}CH_3$	C_{35}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{19}CH_3$	C_{36}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{20}CH_3$	C_{37}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{21}CH_3$	C_{38}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{22}CH_3$	C_{39}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{23}CH_3$	C_{40}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{24}CH_3$	C_{41}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{25}CH_3$	C_{42}
$CH_3(CH_2)_{14}C(=O)-$	$-O(CH_2)_{26}CH_3$	C_{43}

grow on sodium acetate but not on cetyl palmitate. The logic was that while certain mutants would retain their ability to synthesize wax esters, they would have a decreased capacity to degrade them. Cetyl palmitate is a model wax ester. Mutants that fulfilled these requirements were tested in parallel fermentations with the parental strain.

RESULTS AND DISCUSSION

Wax Esters From n-Alkane Mixtures

The data in Table I indicate that the wax esters produced by *Acinetobacter* sp. HO1-N are a function of the chain length of the n-alkane substrate or substrate mixture. The use of individual n-alkanes in the range C_{16}-C_{22} yields wax ester products containing 1 to 3 different C-chain lengths, while the use of n-alkane mixtures yields products with up to 7 different C-chain lengths (see C_{16}+C_{22}). That this flexibility in wax ester product range can be extended far beyond this is illustrated in Table II, wherein the mixture of n-hexadecane (C_{16}) and primary gas oil (C_{15}-C_{30}) yields a wax ester mixture containing at least 13 components, C_{16}-C_X, where x = 15-27.

Effect of n-Hexadecane Concentration on C_{32}-Wax Ester Production

The data in Table III illustrate the finding that increasing the level of n-hexadecane in the wax ester-forming stage increases the yield of wax ester. At each level indicated, there is residual n-hexadecane at the completion of the experiment. Therefore, the stimulating effect of increasing n-hexadecane concentration is not due to the simple availability of substrate. At least 3 reasons can be cited to explain the results:

a) The wax ester product is extracted into the n-hexadecane phase reducing its hydrolysis to fatty acid and alcohol by esterases or lipases produced by *Acinetobacter* sp. HO1-N.
b) The n-hexadecane substrate must be in a particular physical state, such as particle size, for optimal wax ester production, and high levels favor this condition.
c) The biphasic system, n-hexadecane/water, favors the reversal of the extracellular esterase or lipase hydrolysis of wax ester, yielding wax ester rather than component fatty acids and alcohols (8).

Further studies are needed to distinguish among these possibilities.

Improved Mutants for Wax Ester Production

The data in Table IV confirm that increasing the concentration of n-hexadecane results in a high yield of C_{32}-wax ester, both in the parental *Acinetobacter* sp. HO1-N and the mutant CP179-22. In addition, the mutant produced 20-30 times the yield of isolated C_{32}-wax ester at each n-hexadecane level. Mutant CP179-22 was obtained from MNNG treatment at pH 5, followed by 4 hr of expression in Mueller Hinton broth.

It can be seen from the data in Table V that, whereas the parental strain gives the best yields of C_{32}-wax ester at low cell count in the inoculum, the mutant gives best yields at high cell count. At cell counts of 2-3 x 10^7/ml, in this experiment, the strains are roughly equivalent in productivity. One interpretation of these results is that the mutant strain has a lesser capacity to degrade the C_{32}-wax ester, perhaps because of a decrease in degradative esterase or lipase activity. In Tables III and IV, the different yields of wax ester from 0.6 g/L n-hexadecane can be explained by the difference in cell counts of the parental strain in the fermentation at T_O.

With the mutant strain, a maximum yield of 1.10 g/L C_{32} wax from 6.2 g/L n-hexadecane was obtained after 22 hr under similar conditions. Since there was loss of n-hexadecane to the atmosphere during the fermentation and 20% residual n-hexadecane, this ~22% conversion is a minimum value for the study. The parental strain, also having 20% residual n-hexadecane, yielded 0.264 g/L or ~5.3% conversion at 22 hr. The T_O cell count/ml for the mutant fermentation was 7 x 10^8, and that of the parental strain was 2 x 10^7. Further, more controlled studies are necessary

TABLE III

Effect of Increasing Levels of n-Hexadecane on C_{32}-Wax Ester Formation by *Acinetobacter* sp. HO1-N*

n-Hexadecane level (g/L)	C_{32}-Wax ester yield (mg/L 24 hr)
0.16	0.7
0.6	7
3.1	100

*Fermentation cell count = 4 x 10^8 cells/ml at T_O

TABLE IV

Effect of n-Hexadecane Level on C_{32}-Wax Ester Formation by *Acinetobacter* sp. HO1-N and Mutant CP179-22

Strain	n-Hexadecane level 0.6 g/L	6.2 g/L
	wax ester yields (mg/L)	
Parental	0.6	9
Mutant CP179-22	11	274

Parental fermentation cell count = 4 x 10^9 cells/ml at T_O.
Mutant fermentation cell count = 5 x 10^7 cells/ml at T_O.

TABLE V

Effect of Inoculum Cell Number on Wax Ester Production of *Acinetobacter* sp. HO1-N and Mutant CP179-22 From n-Hexadecane*

Strain	cells/ml (wax ester titer, mg/l)	cells/ml (wax ester titer, mg/l)	cells/ml (wax ester titer, mg/l)	cells/ml (wax ester titer, mg/l)
Parental	1.6 x 10^7 (134)	8 x 10^7 (53)	4 x 10^8 (38)	8 x 10^8 (43)
Mutant CP179-22	2 x 10^6 (4)	1 x 10^7 (38)	5 x 10^7 (236)	1 x 10^8 ()344)

*n-Hexadecane at 6.2 g/L, initial concentration. Inocula for this experiment were grown in the modified mineral medium with 0.155 g/L n-hexadecane, the usual fermentation medium described in experimental procedures.

to determine the maximum efficiency for n-hexadecane (and other n-alkanes) conversion to wax ester.

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Developing a New Industrial Enzyme Application: A Strategy

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ABSTRACT

Many companies view industrial enzymes as potential tools for making technological breakthroughs in their industries. Some have created special biotechnology groups to pursue this aim. Too often their projects are terminated before the intended goals are met. A major reason is too much time was consumed for too little perceived gain. A strategy is proposed to help R&D groups and their managers conserve valuable resources. It focuses on the essentials in evaluating new enzyme applications and in better developing discoveries.

ENZYME BENEFITS

Biotechnology—enzymes, in particular—is a new technology for the fats and oils industry. Enzymes are very familiar to those in the detergent and vegetable protein industries. There is much excitement in the business and R&D sectors over the potential for biologically modifying fats and oils for profit. At some point someone will ask, "Are such great things really possible, or is it just 'pie in the sky'?"

The answer to this question is, "Both." That is, there are objectives for which the basic technology exists today, and details must be developed. There are other objectives which will require many years of basic research to ensure success.

Enzymes are definitely "real": enzymes perform valuable tasks in many industries today. Some of these may be readily adaptable to the fats and oils industry. A few examples of how enzymes are benefiting their users can be discussed.

Many enzymes can cause viscosity reductions on materials through hydrolysis. Fishmeal processors recently have learned that a protease can save them money in drying costs (1). The protease is used to hydrolyze the protein in stickwater produced in the Menhaden process. The viscosity reduction that results leads to improved drying efficiency later in the process.

Enzymes are used to degrade the pectin and cellulose in apple juice processing. Here, too, there is a viscosity reduction; this time there is an improvement in filterpressing of the juice from the fruit. More importantly the hydrolysis improves the extraction of the juice (2). This gives the processor more saleable product and increases his return on his feedstock.

One of the most familiar uses of enzymes is in the production of corn syrups. Three enzymes are used to convert corn starch into high fructose corn syrup. In this case enzymes achieve higher conversion to end product with less by-product formation than chemical processes. Also, enzymes give producers the flexibility to make syrups to meet specific customer needs at a competitive price (3,4).

The decolorization of slaughterhouse blood is an example of where an enzyme can affect a separation. In this case a protease extensively hydrolyzes the hemoglobin in the blood. When the pH is lowered, the pigment precipitates. This can be removed using a centrifuge or a filter press. The light colored broth can be used in processed meats in Europe (5,6).

The functional properties of soy protein can be modified using enzymes. Through controlled hydrolysis with proteases soy protein whipping agents can be prepared. The protease causes the soy protein to become more soluble and to have foam expansion and stability after whipping. Thus, enzymes are able to get a value-added return on this vegetable protein (7,8).

Enzymes can be used to affect the flavor of foods. For example, the piquant flavor note in certain Italian cheeses is caused by the action of lipases or pregastric esterases. Also, a key flavor note in cheddar cheese is due to protease action. Law and others have described how enzymes can be added to hasten the cheese aging process (9,10,11). Accelerated cheese ripening saves producers money in inventory costs. Enzyme modified cheese (EMC) is becoming an item of commerce in the flavor industry.

Some hydrolytic reactions can be made to run in reverse in organic solvents. Therefore, some enzymes can be used to synthesize compounds. For example, Strobel et al demonstrated how a crude enzyme preparation could be used to make terpene esters in high yields (12). Another plus in this case is that the enzyme is specific in terms of which stereoisomer is produced, unlike chemical methods which produce racemic mixtures. This is beneficial in the flavor and fragrance industry, where one stereoisomer may be valuable and the other not.

Lastly, amylase and protease enzymes have been added to pre-soak detergents for more efficient cleaning and stain removal (13). Such preparations have been used widely for years in the detergent industry.

Larry Posorske, my colleague, discusses emerging enzyme applications for modifying fats and oils in his paper (14). The rest of this paper will outline a strategy for developing your own enzyme application. It focuses on the essentials in evaluating new enzyme concepts and in developing discoveries into beneficial applications. It will help R&D groups and their managers to conserve valuable resources and to avoid the major pitfalls of enzyme projects.