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Optimized Conditions for the Production of Hydroxamic Fatty Acids from Coconut Oil by a Lipase from *Rhizopus delemar*

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Abstract

A lipase from *Rhizopus delemar* was used as a biocatalyst for the synthesis of hydroxamic fatty acids (HFA) by reacting hydroxylamine with coconut oil, methyl laurate and lauric acid. The effects of various reaction parameters such as amount of hydroxylamine-HCl, incubation time, temperature, and amount of enzyme were evaluated to achieve high product yield.

The reaction products were monitored by Thin Layer Chromatography using hexane: diethyl ether: acetic acid (20:80:1 v/v/v) as solvent system. Identification of the products was done by Gas-Liquid Chromatography. Analysis revealed a mixture of HFA produced using coconut oil as substrate: 30.33% myristylhydroxamic acid (MHA), 24.17% olelyhydroxamic acid (OHA) and 45.50% laurylhydroxamic acid (LHA). On the other hand, methyl laurate and lauric acid yielded only LHA.

Purification of the product was done by crystallization in hexane at 14 °C. Identification of the functional groups present was determined by Infrared (IR) spectroscopy.

Keywords: Coconut oil, hydroxamic fatty acids, *Rhizopus delemar* lipase

Abbreviations: HFA-hydroxamic fatty acids, IR-infrared, LHA-laurylhydroxamic acid, MHA-myristylhydroxamic acid, OHA-olelyhydroxamic acid

Introduction

Hydroxamic fatty acids (HFA) are N-hydroxylated amides (RCONHOH) synthesized by aminolysis of fatty acid ester or reaction between fatty acid and hydroxylamine. These compounds are receiving a lot of attention due to their biological activity as inhibitors of cyclooxygenase and 5-lipoxygenase with potent topical anti-inflammatory activity (Bondarenko et al., 1997, Hamer et al., 1996) and lipoxygenase in potato which control tuber development (Butovich and Reddy, 2002). They also act as chelating agents (Suhendra et al., 2005a), which are capable of removing impurities from mineral ores and may be used in waste water treatment. In addition, due to the amphiphilic nature of these molecules, they can be used as precursors for surfactants with a wide range of properties enabling them to be used in numerous ways such as household cleaning products, cosmetics and pharmacology (Servat et al., 1990a).

One of the pioneers in this area is the group of Servat, who in 1990a began synthesizing HFA from the oleic acid and soybean methyl ester catalyzed by a lipase from *Mucor miehei*. In the same year another group also headed by Servat studied the bioconversion of triglycerides from different oils to HFA. In 1997, Vaysse et al. investigated the biosynthesis of hydroxamic fatty acid in aqueous medium in the presence of the lipase-acyltransferase from *Candida parapsilosis*. The use of immobilized lipase as biocatalyst for the synthesis of HFA from palm oil was successfully reported by Suhendra et al. (2005b).

Chemical synthesis of HFA can be carried out by reacting hydroxylamine with fatty acid esters in the presence of sodium methoxide. According to Servat et al. (1990a), enzymatic synthesis gives higher yield of HFA compared to chemical synthesis. Suhendra et al. (2005b) added that the chemical synthesis, which is in alkali system, could cause the decomposition of the product due to the presence of double bonds.

This paper aims to determine the optimum parameters for the synthesis of HFA from coconut oil, methyl laurate and lauric acid with the use of a lipase from *Rhizopus delemar*, and to characterize and purify the products.

Materials and Methods

A crude lipase from *Rhizopus delemar* was obtained from the Enzyme Laboratory, National Institute of Biotechnology and Applied Microbiology (BIOTECH), University of the Philippines Los Baños, College, Laguna. All chemicals used were analytical grade and purchased from Sigma Chemical Company (St. Louis, U.S.A.) except for methyl laurate which was produced in the Biochemistry Laboratory of the Institute of Chemistry, University of the Philippines Los Baños. Commercial coconut oil used was Minola™ oil which was purchased locally.

Enzymatic synthesis of HFA

In a screw-capped flask, which served as a reaction vessel, 0.5 mL coconut oil was added to 2.0 g hydroxylamine-HCl (NH_2OH-HCl) neutralized in 4 N NaOH, and 0.5 g enzyme in 3 mL 0.05 M phosphate buffer. The flask was incubated in a water bath shaker for 24 h at 40 °C. The product formed was extracted with 10 mL hexane leaving behind the enzyme in the aqueous layer.

The same procedure was employed using 0.2 g lauric acid in 5 mL hexane and 0.5 mL methyl laurate as substrates.

Optimization of conditions

The optimum conditions were determined by varying the amount of NH_2OH-HCl (0.5, 1.0, 1.5, 2.0 and 2.5 g), incubation time (6, 12, 24 and 36 h), temperature (30, 40, 50 and 60 °C), and amount of crude enzyme (0.25, 0.5, 0.75 and 1.0 g).

Quantification of HFA

The products were colorimetrically quantitated in hexane as described by Servat et al. (1990a). Three milliliters of HFA solution was added to 0.5 mL $FeCl_3$·6H2O (6% w/v in 2 N HCl) then stirred. The absorbance was read at 450 nm in comparison with the control using the spectrophotometer (Beckman DU-650, U.S.A.).

Determination of the fatty acid composition of the product

1. Thin layer chromatography

Products were spotted on a 0.25 mm thick, ready-to-use silica gel plates (Merck 60G), developed by a hexane: ethyl ether: acetic (20:80:1 v/v/v) mixture and sprayed with a solution containing a mixture of saturated copper acetate in water and 85% phosphoric

acid (50:50 v/v). After the plates have been oven-dried at 120 °C, the products were visualized in daylight, and the bands were scraped off. The products fixed on silica gel were desorbed in ethyl ether.

2. Esterification

After evaporation of ethyl ether, the HFA were directly transformed into methyl esters by addition of 4 mL methanolic-HCl. The mixture was then incubated at 70 °C for 12 h and cooled at room temperature. The methyl esters were extracted with hexane.

3. Gas-liquid chromatography

The methyl esters were analyzed with a gas chromatograph (Shimadzu GC-14B, Japan) equipped with Flame Ionization Detector (FID) with BP-20 column. Two milliliter sample was injected every 30 min running time. Identification of the products was done by comparing the retention time of a standard. Experimental conditions used are: column temperature: 90-190 °C, injector temperature: 230 °C, detector temperature: 240 °C, program rate: 4 °C·min^{-1}

Purification and identification

The hydroxamic fatty acids were purified according to the method described by Servat et a1. (1990a). The product extracted from the reaction mixture was crystallized at 14 °C. After purification, the degree of purity was confirmed by IR spectroscopy. The samples were submitted for IR analysis to the Forest Products Research and Development Institute of the Department of Science and Technology at the University of the Philippines Los Baños, College, Laguna for IR analysis.

Statistical analysis

The experiments were done with the different levels of hydroxylamine·HCl, incubation time, temperature and enzymes as treatments, each experiment with three replicates. Results were statistically analyzed using Complete Randomized Design. Duncan's Multiple Range Test was used for mean separation.

Results and Discussion

Enzymatic synthesis of HFA

Crude lipase preparation from *Rhizopus delemar* was used as a catalyst for the synthesis of HFA. The enzyme had enzyme activity, specific activity and protein content of 5.61 μmole· (min·mL)$^{-1}$, 0.239 U·mg^{-1} protein and 23.40 mg·mL^{-1}, respectively. One enzyme unit (U) is defined as the amount of lipase that released micromoles of free fatty acid per unit time.

When the coconut oil, lauric acid and methyl laurate were brought in contact with hydroxylamine-HCl neutralized with 4 N NaOH, two immiscible layers were formed. The top layer, which is the organic layer, contains the triglycerides, methyl esters or fatty acids, depending on the type of substrate used. The bottom layer would constitute the hydroxylamine and the enzyme in phosphate buffer. When lauric acid in hexane was brought in contact with hydroxylamine-HCl, a viscous medium was produced due to the insolubility of the fatty acid. As the reaction proceeded, the mixture turned cloudy while the control remained clear indicating that a reaction had taken place. In the course of the reaction as cited by Servat et al. (1990a), the hydroxyl ammonium soaps probably arranged themselves in mixed surfactant-free fatty acid micelles. The arrangement of free fatty acids in this type of macrostructure exposes their carboxylic part to the surface of the micelle. This organization facilitated the formation of an acyl enzyme; an intermediate subsequently evolving into a HFA by reaction with hydroxylamine-HCl. Fournand et al. (1997) and Vaysse et al. (1997) reported the possible pathway for the biosynthesis of HFA as shown in Figure 1.

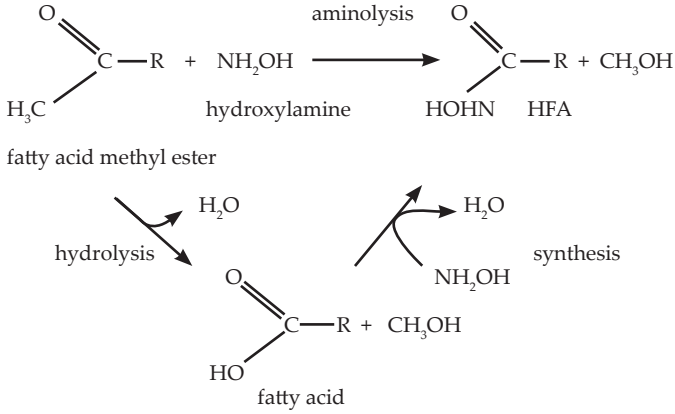

Figure 1. Possible reactions for the synthesis of hydroxamic fatty acids (HFA)

Quantitative analysis of the product was done using the colorimetric technique developed by Servat et al. (1990a). This method takes advantage of the propensity of HFA to chelate ferric ions in hexane medium. Hydroxamic fatty acids react with ferric

chloride in acid solution to form soluble ferric hydroxamates that are highly colored. The color of this salt is bluish red (magenta) or a deep red color is often noted particularly if the concentration is high. Intensity of the color is directly proportional to the concentration of HFA in the solution.

Two layers were formed as a result of this reaction. The top layer, the organic phase, contains ferric hydroxamate which is red in color. The bottom layer, which is the aqueous layer, contains the unreacted ferric chloride.

Effect of varying amounts of hydroxylamine hydrochloride

The effects of different amounts of hydroxylamine-HCl (NH_20H-HCI) on HFA synthesis were investigated using coconut oil, methyl laurate and lauric acid as substrates. There was a rapid increase in the production of HFA when the amount of reagent was doubled from 0.5 to 1.0 g using methyl laurate and lauric acid as substrates. A continuous increase in the production of HFA was observed when coconut oil was reacted with 1.5 g NH_2OH-HCl (Figure 2). Further increase in the amount of coconut oil did not cause any significant change, indicating that equilibrium has already been attained. The high level of HFA obtained using coconut oil is attributed to the fact that it contained a mixture of fatty acids compared to the other two substrates which purely contained lauric acid.

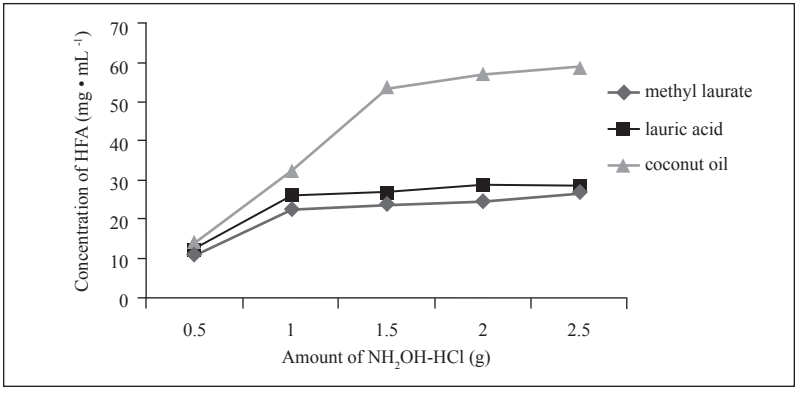

Figure 2. Effect of increasing amount of NH2OH-HCl on the synthesis of hydroxamic fatty acids (HFA) from different substrates. Methyl laurate ($F= 91.04$, $p < 0.05$), lauric acid ($F= 39.27$, $p < 0.05$), coconut oil($F= 89.60$, $p < 0.05$).

Effect of incubation time

Time course is a good indicator of enzyme performance and progress of reaction. It helps determine the time necessary to obtain good yield, thereby making the process cost-effective (Yee and Akoh, 1996). Formation of HFA was monitored with varying incubation times (6, 12, 24, and 36 h) at 40 °C. HFA concentrations increased with incubation time in all substrates (Figure 3). A significant increase in the yield of HFA was observed when the mixture was allowed to react up to 12 h using methyl laurate and lauric acid as substrates. However, with coconut oil as substrate, production of HFA continued to increase when the mixture was incubated for 24 h. No significant changes were observed when the reaction time was extended. It took longer for the reaction using coconut oil as substrate to achieve optimal HFA concentration. This was because the fatty acids in the triglyceride molecule should be first cleaved before it could be used for the synthesis of HFA. Unlike in lauric acid and methyl laurate, the triglyceride molecule could be readily acted by hydroxylamine. Servat et al. (1990a) reported that the production of oleylhydroxamic acid proceeded more rapidly as incubation time increased. After 24 h reaction, the production of HFA slowed down.

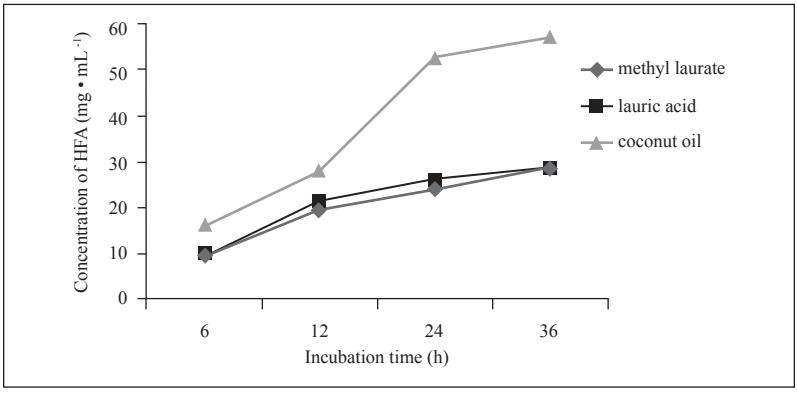

Figure 3. Enzymatic synthesis of hydroxamic fatty acids(HFA) over time from different substrates. Methyl laurate ($F = 36.09$, $p < 0.05$) lauric acid ($F = 75.92$, $p < 0.05$), coconut oil ($F = 119.35$, $p < 0.05$).

Effect of temperature

The dependence on temperature of lipase, which catalyzed synthesis of HFA, was investigated at 30, 40, 50 and 60 °C. As shown in Figure 4, the optimum temperature was shown to be 40

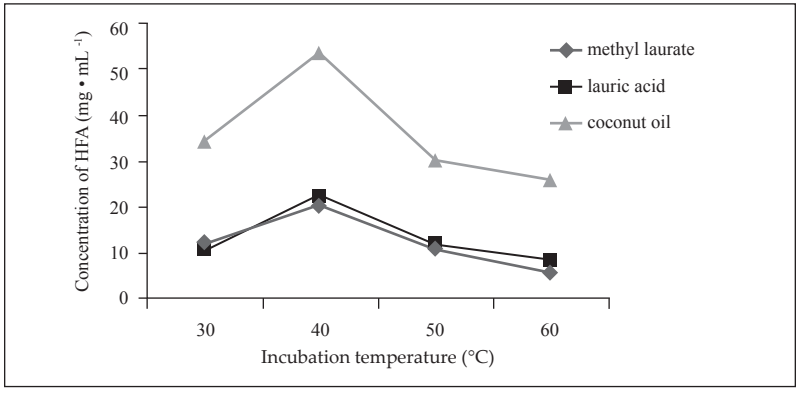

Figure 4. Dependence of hydroxamic fatty acids (HFA)yield from different subtrates on incubation temperature. Methyl laurate ($F= 24.48$, $p < 0.05$), lauric acid ($F= 39.43$, $p < 0.05$), coconut oil ($F= 44.69$, $p < 0.05$)

°C regardless of the type of substrate used. When the temperature was further raised to 50 °C, a sudden drop in the concentration of HFA was observed which continued to decline at 60 °C. Reaction temperature has a profound influence on the reaction rate (Pawongrat et al., 2007). As the temperature is increased reaction rate continues to rise until it reaches the optimum temperature. The degree to which the temperature is increased is limited by the fact that extreme temperatures will completely and irreversibly denature the lipase (Fomuso and Akoh, 2002). As a result the lipase loses its activity which is noted by a decrease in HFA synthesis at temperatures beyond 40 °C.

Effect of amount of enzyme

With the high cost of enzymes, the amount of enzyme used is crucial for the enzymatic process to be competitive with the chemical process. Results showed no significant changes in the synthesis of HFA from methyl laurate and lauric acid despite an increase in amount of enzyme used (Figure 5). In the case of coconut oil, the reaction rate proceeded rapidly as the amount of enzyme was doubled from 0.25 to 0.5 g. Further increase in the amount of enzyme did not cause a significant rise in the concentration of HFA. This indicated that equilibrium had already been attained. According to Gandhi et al. (1995), the reaction rate is proportional

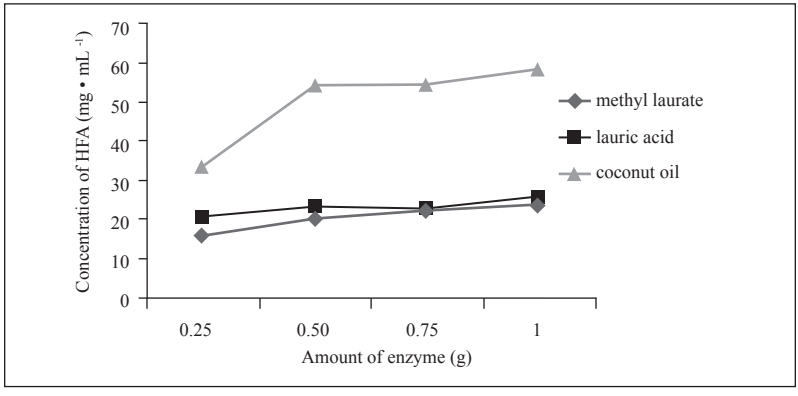

Figure 5. Effect of varying amount of enzyme on the yield of hydroxamic fatty acids (HFA) from different substrates. Methyl laurate ($F = 4.54$, $p > 0.05$), lauric acid ($F = 1.44$, $p > 0.05$), coconut oil ($F = 35.51$, $p < 0.05$).

to the number of active sites available. Hence, the reaction is a function of the interfacial area. However, once the interfacial area is completely filled with the available enzyme molecules, any further increase in the enzyme concentration would only push the extra lipase molecules into the interior of the aqueous area. This explains why further addition of the enzyme did not cause any significant change when the optimum condition has been reached.

Characterization of the product

The optimum parameters obtained were used in the synthesis of HFA. With lauric acid as substrate, the conditions used were 0.2 g lauric acid, l.0 g hydroxylamine-HCI, and 12 h incubation time at 40 °C using 0.25 g crude enzyme. The same conditions were used using 0.5 mL methyl laurate as substrate. For coconut oil, the following conditions were used: 0.5 mL coconut oil, l.5 g hydroxylamine-HCl, and 24 h shaking at 40 °C using 0.5 g enzyme. Two spots were noted regardless of the type of substrate used. Spots were marked upon their appearance since the color immediately disappears after one minute. Each unknown component was identified based on the comparison of the retardation factor or Rf value of the sample with that of the standard. Triglycerides and methyl esters migrated to the solvent front while free fatty acids and HFA had Rf values of 0.8 and 0.5, respectively.

Gas-Liquid chromatographic analysis revealed that LHA was the product formed from lauric acid and methyl laurate. With coconut oil as substrate, mixtures of HFA were produced: LHA (45.50%), MHA (30.33%) and OHA (24.17%). Based on the chromatogram, LHA, MHA and OHA had retention times of 13.65, 19.28 and 24.62 min, respectively. Furthermore, results showed a high percentage of LHA due to high lauric acid content in coconut oil.

In the study done by Servat et al. (1990b), the fatty acids of the HFA synthesized from coconut oil ranged from C6 to C18:2. Aside from coconut oil, they also used other oils such as palm, olive, cod liver and myrianthus oil in the synthesis of HFA. All the fatty acids present in the said oils were used in the synthesis. In this study, only lauric (C12:0), myristic (C14:0) and oleic (C18:1) acids were used. This difference could be due to the fatty acid specificity of the enzyme used.

Infrared (IR) analvsis

The HFA produced were purified prior to IR analysis. Partial purification was achieved by crystallization in hexane at 14 °C. The resulting product was a fine white substance which remained solid at 14 °C and turned to liquid at room temperature.

IR analysis revealed similar bands in the infrared spectrum of coconut oil and the synthesized HFA, except that a band was noted at 3275.70 cm^{-1} frequency in the HFA spectra (Figure 6). This band corresponds to N-H bond which confirms the formation of hydroxamic fatty acid in the reaction.

Conclusions

Enzymatic synthesis of HFA was carried out by reacting hydroxylamine with fatty acids in their free or methyl ester forms. Lauric acid, methyl laurate and coconut oil were used as substrates. The effects of the amount of hydroxylamine-HCI, incubation time, temperature, and amount of enzyme on HFA synthesis were studied. The optimum operating conditions noted for the production of HFA from coconut oil were: 1.5 g hydroxylamine, 24 h incubation time at 40 °C using 0.5 g enzyme, while for methyl laurate and lauric acid, the optimum conditions observed were: 1.0 g hydoxylamine-HCI, 12 h at 40 °C using 0.25 g enzyme. Laurylhydroxamic acid (LHA) was the common product for all substrates. In coconut oil as substrate, however, mixtures of HFA were formed: 45.50% LHA,

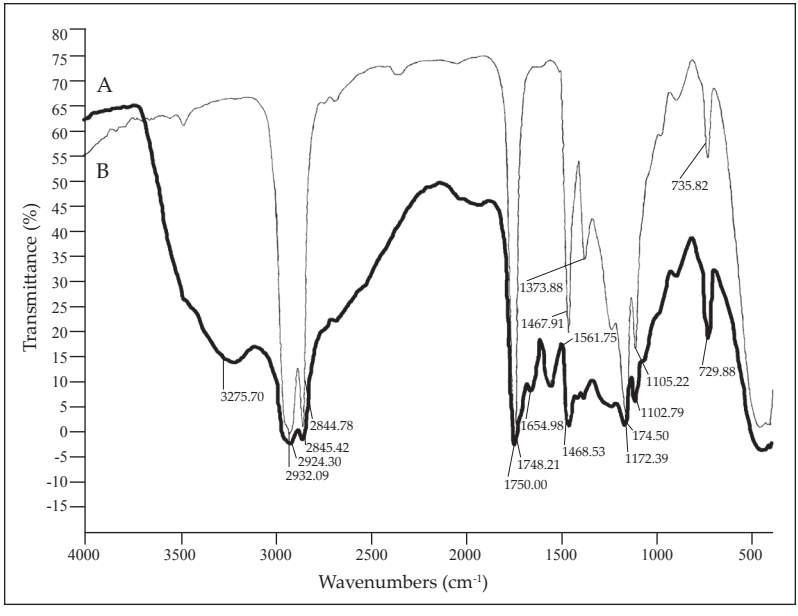

Figure 6. IR spectrum of (A) coconut oil and hydroxamic fatty acids (HFA) from coconut oil (B)

30.33% MHA and 24.17% OHA. Only lauric, myristic and oleic acids from coconut oil were used in the synthesis due to the substrate specificity of the enzyme

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