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Biocatalytic transformation of steroids using solvent-enhanced Beauveria bassiana

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BIOCATALYTIC TRANSFORMATION OF STEROIDS USING SOLVENT-ENHANCED *BEAUVERIA BASSIANA*

by

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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Para mami, papi, bebé, familia y amigos.

The essence of optimism is that it takes no account of the present, but it is a source of inspiration, of vitality and hope where others have resigned; it enables a man to hold his head high, to claim the future for himself and not to abandon it to his enemy.

Dietrich Bonhoeffer

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ABSTRACT

This dissertation describes efforts to improve the oxidative capacity of n-alkane-induced *Beauveria bassiana*; a fungus and a versatile whole cell biocatalyst used in the biotransformation of steroids. n-Hexadecane was used as the carbon source during the growth of *B. bassiana*, presumably to induce the expression of oxidative enzymes, thus enhancing the oxidation of unactivated carbons. Dehydroepiandrosterone (DHEA) is an essential endogenous male-hormone and serves as a metabolic intermediate in the production of more potent androgens. Using DHEA as a substrate also provides the opportunity to study the hydroxylation of an unfunctionalized carbon, an attractive reaction that produces valuable intermediates for chemical synthesis. Results showed that exposing and inducing cells in n-hexadecane improves the synthesis of 11α-hydroxy derivatives. Reactions were carried out with cells grown on n-hexadecane, resulting in 65 ± 6.3 % conversion of DHEA to androstenediol (40.3% mM) and 3β,11α,17β-trihydroxyandrost-5-ene (22.8% mM), as determined by HPLC, NMR and LCMS analyses. However, experiments with non-induced cells resulted in a poor substrate conversion (17%). To extend use of *B. bassiana* to pharmaceutical applications, it was necessary to optimize reaction conditions such as biocatalyst preparation, substrate concentration, agitation reaction temperature and pH. Higher substrate conversion, selectivity and yield of desired product were achieved with the reactor arrangement of "Resting Cells". The apparent rate of reaction fits a Michaelis-Menten kinetic model with a maximum reaction rate of 4.45 mM/day, revealing that the transformation of intermediate androstenediol to desired 3β,11α,17β-trihydroxyandrost-5-ene is the limiting

step in the reaction. Interestingly, when a diluted amount of substrate was used, a higher yield of 11α-hydroxy steroid was achieved. Also, reactions at 26°C with pH ranges between 6.0 and 7.0, resulted in the highest conversion (70%) and the higher product yield (45.8%). The maximum conversion of DHEA (71%) was achieved in experiments with high biomass loading, and the increment of desired product yield (11α-hydroxy) was directly proportional to the amount of biomass used. Moreover, a high V_{Max}/K_M value was achieved with high biomass yields. Interestingly, the changes in biomass yield did not have a considerable effect on reaction selectivity. The main drawbacks of biocatalysis for production of steroids were addressed and approaches to minimize the drawbacks have been presented. The production of desired product (11α-DHEA) was significantly improved using cells previously adapted to n-hexadecane.

PUBLIC ABSTRACT

This dissertation describes efforts to improve the oxidative capacity of *Beauveria bassiana*, a fungus and a versatile whole cell biocatalyst used in the biotransformation of steroids. Organic solvents such as n-hexadecane were used as the carbon sources during the growth of *B. bassiana*, presumably to induce the expression of oxidative enzymes, thus enhancing the oxidation of unactivated carbons. These types of oxidations are important in the synthesis of steroids, which are used as anti-inflammatory, diuretic, anabolic and contraceptive agents. In this work, we used dehydroepiandrosterone (DHEA), an essential male-hormone that serves as an intermediate in the production of more potent steroids. The use of DHEA as a reactant also provided the opportunity to study the hydroxylation of an unfunctionalized carbon. Hydroxylated steroid derivatives are valuable intermediates in industry since they cannot be easily synthesized by solely chemical means. The oxidative performance with solvent-enhancement resulted in better biocatalyst control over product distribution. This enables the application of enzyme expression to product specifications with more expensive substrates. To extend use of *B. bassiana* to pharmaceutical applications, it was necessary to optimize reaction conditions such as biocatalyst preparation, substrate concentration, agitation reaction temperature and pH. Results show that exposing cells to n-hexadecane improves the synthesis of 11α-hydroxy derivatives. The main drawbacks of biocatalysis for producing steroids were addressed, and approaches to minimize the drawbacks have been presented. The production of the desired product (11α-DHEA) was significantly improved using cells that are previously adapted to n-hexadecane.

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PREFACE

B. bassiana is widely used in organic chemistry to perform biotransformations due to its biocatalytic power and simplicity to handle. This dissertation describes efforts to improve the oxidative modification of unfunctionalized carbons as result of virulence enhancement conditions. Reaction optimization and accurate prediction of products are necessary to enhance the use of *B. bassiana* in steroid synthesis for anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, progestational and anticancer agents.

n-Dodecane and n-hexadecane are used to induce gene expression of oxidative cytochrome P-450 enzymes, and the expression of these enzymes highly depends on the n-alkane they oxidize. An enzyme-induction system was designed for selective hydroxylation and to facilitate cost effective and eco-friendly production of steroids that cannot be easily synthesized by solely chemical means.

This research targets the 11α-hydroxylation of DHEA, since this molecule provides a reaction site for the hydroxylation of an unfunctionalized carbon. With this feature and its cost ($9.6/g), DHEA appears as a promising substrate for optimization experiments. The optimal experimental parameters determined with this substrate can be correlated for the activation of unactivated carbon on other more expensive substrates. The goal of this research will be achieved by meeting the following objectives:

- Enhance the oxidative performance of *B. bassiana* for the biotransformation of steroid DHEA using suitable n-alkane solvents as carbon source,
- Use HPLC, NMR AND LCMS to evaluate conversion, yield and selectivity of biotransformation with cells induced and adapted to n-alkanes,

- Correlate kinetic parameters and biomass yields of cells adapted to n-alkanes.

This research provides a cost effective way to study the effect of organic solvents on the synthesis of steroid intermediates. This work has enabled the development of a more robust biocatalyst with higher biohydroxlyation capacity. Results obtained support biotechnology processes engaged in the production of expensive drugs. This will ultimately lead to a well-characterized biocatalyst with transformative potential to overcome technology gaps for selective bio-oxidations of commercial interest.

CHAPTER 1
INTRODUCTION

Specific Aim and Hypothesis

The long-term goal of this research is to standardize, control and optimize the biocatalysis capacity of *Beauveria bassiana* in the biotransformation of chemicals operating with different reaction parameters. This research links agricultural biotechnology and biocatalysis, in effort to develop tunable biotransformation processes for selective oxidation.

The principal hypothesis is that enhancements in biopesticide efficacy with n-alkane solvents have synergistic impacts on the biocatalytic inventory of oxidative enzymes within the fungus. Increased oxidative capacity will result in greater biotransformation potential to prove this premise. Chapters 2, 3, and 4 discuss in detail the scope, experiments performed and achievements related to the objectives 1, 2, and 3 respectively.

Objective 1. Enhance the oxidative performance of *B. bassiana* for the biotransformation of steroid trans-dehydroandrosterone (DHEA) using suitable n-alkane solvents as carbon source.

Objective 2. Evaluate conversion, yield and selectivity of biotransformation with cells induced and adapted to n-dodecane and n-hexadecane.

Objective 3. Correlate kinetic parameters and biomass yields of cells adapted to n-alkanes.

n-Alkanes serve as carbon sources during growth of *B. bassiana* in the epicuticle outer layer of insects, and these solvents induce the expression of cytochrome P-450 oxidative enzymes (Sanglard 1989). These enzymes enhance the degradation of hydrocarbons and improve the hydroxylation of unactivated carbons on steroids. Xiong et al. (2006) established a valid steroid biotransformation protocol with *B. bassiana,* and Pedrini et al. (2010) examined the expression of a group of eight hydrocarbon-assimilating P-450 enzymes with specificity to lipids while growing B. bassiana in the presence of n-alkane solvents. Therefore, combining both methodologies, cells will express oxidative P-450 enzymes after induction in a hydrocarbon culture that mimics insect epicuticle, and these enzymes will catalyze steroid hydroxylation. Strategies designed to enhance the efficacy of biopesticides were applied to induce and improve biocatalytic oxidations. This work reports the analysis of bioconversion, oxidative phenotypes and cultivation conditions for biocatalysis with the objective to optimize the bioprocessing scheme.

Background

Beauveria bassiana

B. bassiana is a whole-cell biocatalyst used to perform transformation of chemicals due to its versatile biocatalytic power and simplicity to handle (Huszcza 2005).

The major application of *B. bassiana* has been as a biocontrol agent in the pesticide industry. This entomopathogenic fungus has been applied globally as an environmentally friendly mycoinsecticide (Pedrini 2007). As a result, most work in understanding the complex cellular functions has been in the area of virulence enhancement for pest control, and the impact of oxidative biocatalysis enhancement has not been fully explored. Researchers in the area of green chemistry including our group have applied *B. bassiana* and similar fungal systems in the synthesis of chemicals through selective oxidations. *B. bassiana* is the second most frequently used fungal biocatalyst. Only microbes such as *Aspergillus niger, Pseudomonas putida,* and baker's yeast are applied more frequently (Lehman 2001 and Guanatilaka 2009). There are thirty-four registered strains in the American Type and Culture Collection (ATCC) that differ in their function and isolation. Strains applied as biocontrol agents have been useful in controlling a variety of crop pests. Strains that have been used as insecticides are: *B. bassiana* GHA (Laverlam International Corporation) and ATCC 74040 (Troy Bioscience). *B. bassiana* ATCC 7159 has been the most commonly used strain for biotransformation (Xiong 2006 and Osorio-Lozada 2008). It can catalyze a variety of reactions including: hydroxylations, oxidations, sulfoxidations, glucosidations, acetylations, epoxidations, esterification and lactonization (Baeyer-Villiger) (Holland 1999, Protiva 1968 and Mahato 1997).

Optimization of reaction conditions to increase yields and productivity as well as accurate prediction of biotransformation products are necessary to extend the use of *B. bassiana* in commercial applications.

n-Alkane induction

B. bassiana initiates pest control upon contact with the insect cuticle. It generates a variety of enzymes that facilitate its growth and leads to the death of the insect host (Pedrini 2009). *B. bassiana* biologically attacks the surface layers of the insect host, produces hydrocarbon-assimilating enzymes and uses these hydrocarbons as carbon sources during growth in the insect epicuticle (See Figure 1) (Crespo 2002). Researchers have demonstrated that organic solvents like n-alkane in a culture medium can serve as the carbon source for *B. bassiana.* Pedrini et al. examined the expression of hydrocarbon-assimilating enzymes with respect to n-alkane solvents and found that *B. bassiana* thrives better in the presence of n-hexadecane (n-C_{16}) (See Figure 2) (Pedrini 2007 and 2010).

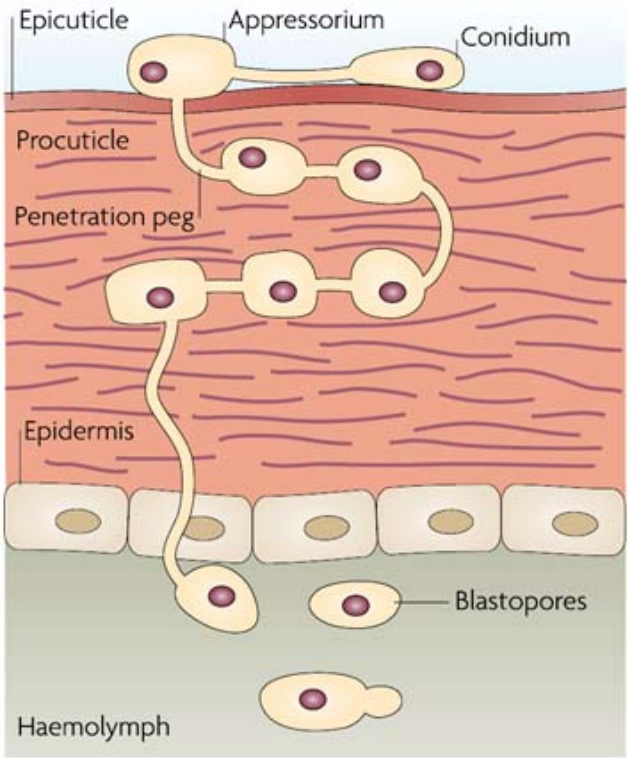


Figure 1. Illustration of growing mechanisms of cells on insect epicuticle surface. This layer serves as protection against biological attacks and is composed by fatty acids, alcohols, waxy esters and n-alkanes (Nature Reviews, Microbiology).

The mechanism of n-alkane degradation by bacteria is well known, but it is not well described for *B. bassiana* (and other fungal strains) (Scheller 1998, Tanaka 1993 and Yadav 1999). Researchers have discovered that the first step in hydrocarbon metabolism based on the bacterial pathways, is the activation of the n-alkane. This is the oxidation of the terminal carbon, which creates a primary alcohol. The second step is the further oxidation of the molecule by aldehyde and alcohol dehydrogenases expressed in *B. bassiana*. This terminal oxidation results in fatty acids that enter the β-oxidation metabolic pathway. If a secondary n-alkane alcohol is produced, it can be converted to ketones that will be further oxidized and hydrolyzed to an alcohol and a fatty acid. Finally, the molecule produced (the fatty acid, or the fatty alcohol) will have several continuous transformations by an alcohol dehydrogenase, an aldehyde dehydrogenase and an acetyl-CoA synthase, to provide the desired fatty acyl-CoA for complete β-oxidation pathway (Pedrini 2007).

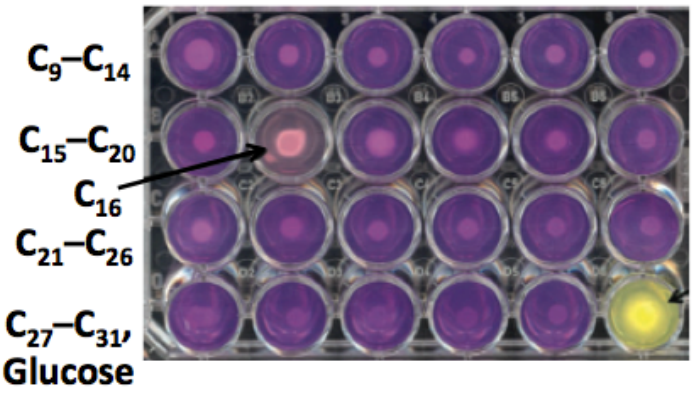


Figure 2**:** *B. bassiana* growth in center of petri dishes with n-alkanes n-C_9 to n-C_{31} as the sole carbon source. Higher cell density was found in n-C_{16} (suitable n-alkane solvent for *B. bassiana* growth). A brighter color is found in the center, compared to growth on glucose (bottom, right) (Pedrini 2010).

Cytochrome P-450 Monooxygenases (CYP)

There are 83 CYP genes encoded in the *B. bassiana* genome and the expression is highly dependent of the presence of the substrate to be oxidized. Until exposure to the substrate, the oxidases remain at their normal levels of expression (Gotoh 1993). These CYPs may be involved in detoxification, degradation of xenobiotics, secondary metabolic pathways or biosynthesis. There are two principal enzyme arrangements in *B. bassiana* for the control of n-alkane oxidation: the monooxygenase that produces the consequent alcohols and the dioxygenase that produces the eventually reduced n-alkyl peroxide. In addition, CYP monooxygenases work in parallel together with a family of NADPH cofactors to generate various monooxidation products (Blomquist 1987, Holland 1999, Holder 2005 and 2007). This demonstrates that the type of enzyme being expressed by the hydrocarbons is crucial in the n-alkane degradation process.

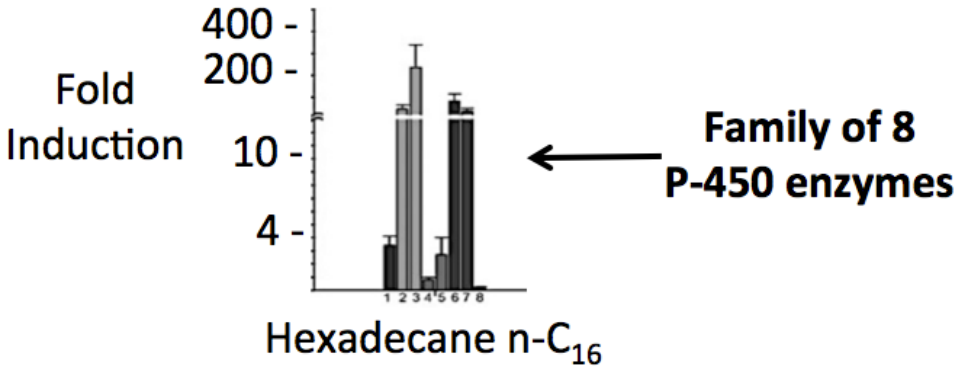


Figure 3: *B. bassiana* oxidative enzyme gene expression during growth on n-hexadecane (n-C_{16}), normalized to gene expression with growth on glucose. Columns represent a family of eight expressed CYP (Pedrini 2010). Values are means ± Std Dev.

It was found that n-alkane solvents induce gene expression of oxidative cytochrome P450 enzymes (CYP). This gene expression varies with the number of carbons in the n-alkane solvent. Pedrini et al. found that *B. bassiana* expresses eight CYP

enzymes that are thought to be involved in the hydrocarbon consumption through β-oxidation pathways (See Figure 3) (Crespo 2000).

Steroids

Steroids are a group of organic compounds whose distinctive arrangement is seventeen carbons in four-rings connected together from three six-carbon rings followed by a 5-carbon ring (For example, see Figure 4.). Hundreds of steroids are synthesized by biochemical processes in animals, fungi and plants, and many steroids are necessary to life at all levels. Steroids include cholesterol, estradiol, bile acids and testosterone.

Applications: Steroids are used as anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, progestational and anticancer agents. In addition, there is potential for expanded applications based on the activation of unfunctionalized carbons of related substrates. The value of microbial biotechnology in the production of steroid drugs was realized for the first time in 1952 when the process of 11α-hydroxylation of progesterone by *Rhizopus* species was patented by Murray and Peterson (Patterson 1952).

Importance: Hydroxylated steroid derivatives are valuable intermediates in industry due to the skeleton provided to synthesize other steroidal drugs (Vardanyan and Andor 2006). Oxidative performance studies with n-alkane solvent-enhancement will lead to better control over product distribution. This enables the application of enzyme expression to enable costly product specifications.

The purpose of this research is to engineer a biocatalytic system that will hydroxylate unfunctionalized carbons during the conversion of steroids to more valuable compounds. Some compounds that require a great amount of effort to synthesize can be easily produced with the use of microbial transformations. This work combines specific biotransformation stages to *optimize* synthesis of essential steroid intermediates for drug development. The Olivo and Peeples team has performed hydroxylations of azibicycloalkanes, N-piperidinylacetophenone and sulfoxidation of modafinil precursors with *B. bassiana* (Osorio-Lozada 2008).

Target substrate: Dehydroepiandrosterone (DHEA)

The activity of *B. bassiana* is being evaluated using 3β-hydroxyandrost-5-en-17-one (DHEA) as substrate. DHEA is a steroid hormone produced from cholesterol by the adrenal glands and is the most abundant hormone in humans (Swizdor 2011). DHEA is metabolized to androgens and estrogens (male and female hormones respectively), and primarily functions as an endogenous precursor to potent androgens like androstenedione, testosterone, estradiol and estrone (See Figure 5.) (Coung 1983).

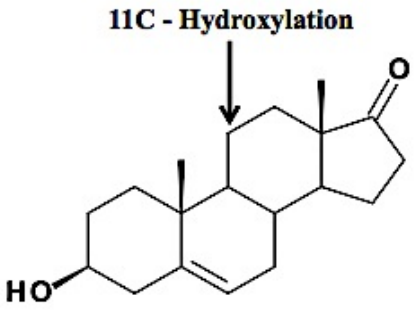


Figure 4: Reaction site of interest in DHEA.

DHEA levels in humans peak at age 25 and then decrease gradually with age. Levels of DHEA in individuals older than 70 are 80% less than in young adults. Researchers have speculated that a low level of DHEA is associated with osteoporosis, heart disease, and memory loss (University of Maryland 2013). Low DHEA levels result in deficiency of essential steroids and the need for the patient to consume synthesized steroid drugs (Capek 1966, Rahim 1966 and Hanson 2003) (See Figure 5.). This research strengthens the case for synthesis of DHEA derivatives and valuable intermediates for medical supplements and patient treatment. An enzyme-induction system is desirable for selective oxidation and eco-friendly techniques for the production of steroids that are difficult to synthesize by classic chemical processes.

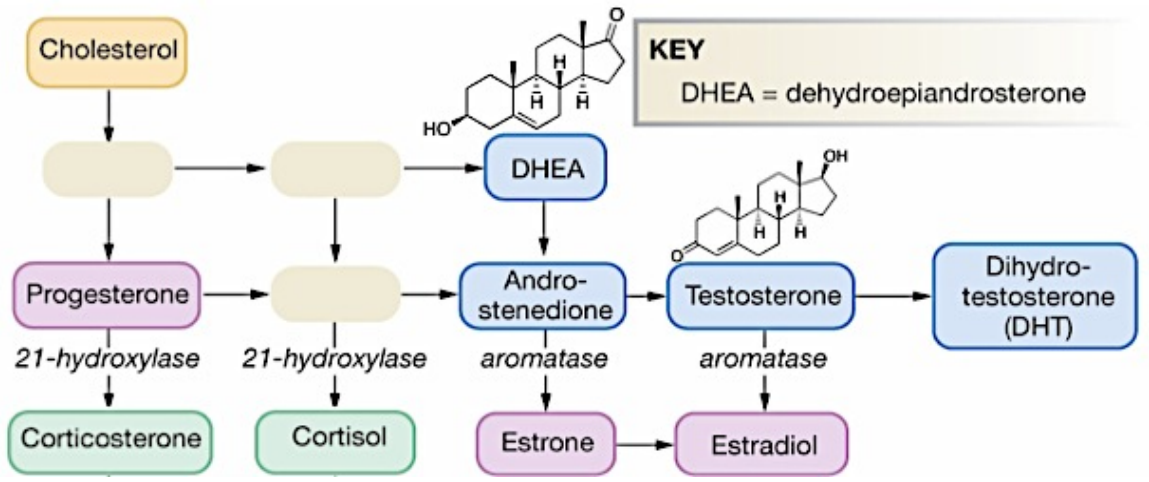


Figure 5: Part of the Adrenal Cortex Pathways in steroid hormone biosynthesis. DHEA (top right) serves as androgens precursor (Chen 2013).

This research targets the hydroxylation of carbon 11 since the structure of DHEA provides a reaction site for the hydroxylation of an unactivated carbon (See Figure 6.). With this feature and its cost ($9.6/g), DHEA is a promising substrate for studies of activation of unfunctionalized carbons. The optimal experimental parameters determined

with this substrate can be correlated for the activation of unfunctionalized carbon on other, more expensive substrates.

The DHEA bioconversion has not been studied with the use of n-alkanes for the induction of oxidative enzymes (Kolek 2008, 2009 and Huang 2010). This work aims to develop a clearer understanding of the oxidative modification of unfunctionalized carbons of steroids as a result of virulence enhancement conditions. The proposed reaction scheme results in 11α-hydroxy-DHEA derivatives using cultivation conditions that induce oxidative enzyme expression. n-Alkane induced cells were used to probe specificity towards DHEA.

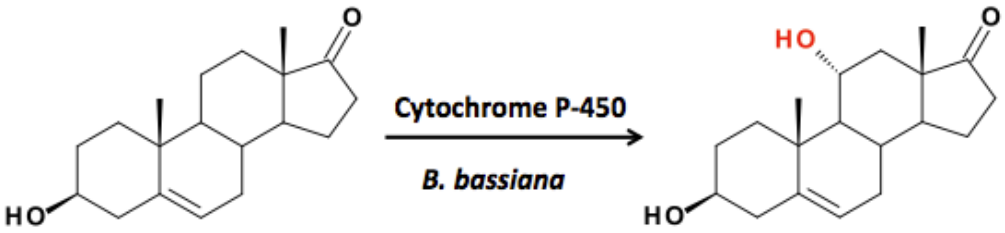


Figure 6: Swizdor et al achieved DHEA to 11α-hydroxylation pathway in low yields. After 48 hours DHEA biotransformation, there was isolated (mol %): (7%) of 11α-hydroxy-DHEA (2) (Swizdor 2011).

Related Substrates

Steroid research is regulated by the "Implementation of the Anabolic Steroid Control Act". The Drug Enforcement Agency (DEA) restricts the use of anabolic steroids like androstenedione without prescription (FR Doc 05-23907). The applications of this work can be useful for the substrates recommended below. These structures allow the hydroxylation of unactivated carbons to occur with cytochrome P-450 enzymes.

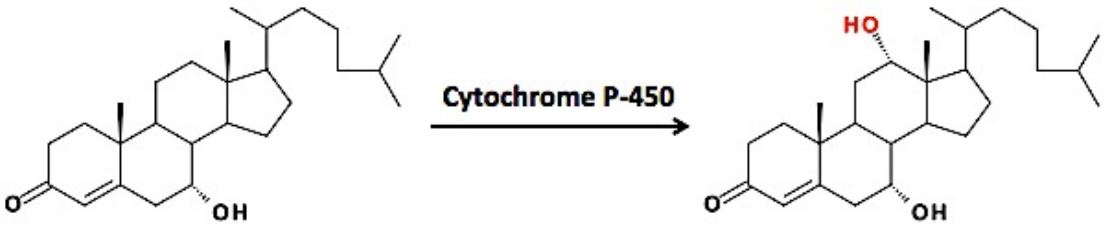


Figure 7: Biotransformation of 7-alpha-hydroxy-4-cholesten-3-one (AHC) to 7-alpha-12 alpha-dihydroxy-4-cholesten-3-one (ADHC).

Dr. Mani V. Subramanian recommended the use of 7-alpha-hydroxy-4-cholesten-3-one (AHC) as a substrate since a private company showed interest in this particular reaction. AHC costs $500/mg and is used for bile acid synthesis with cytochrome P-450 CYP8B1 enzyme. The 12α-hydroxylation involves hazardous chemicals and results in 7-alpha-12-alpha-dihydroxy-4-cholesten-3-one (ADHC) (See Figure 7), which costs $3,150/mg. The balance between these two steroids regulates the relative amounts of cholic acid and chenodeoxycholic acid, both of which are secreted in the bile and affect the solubility of cholesterol (NCBI Homo sapiens cytochrome P450). Deficiency of ADHC causes bile acid malabsorption, Crohn's syndrome (gastrointestinal disease) and Erythema nodosum. The possible low-cost production of ADHC can be achieved with P-450 monooxygenases induced with n-alkanes, thus creating an affordable drug for patients who suffer from bile acid malabsorption. ADHC is synthesized in a process with saturated ammonium sulfate (SAS) precipitation followed by dialysis against phosphate buffered saline (PBS) (Holst 2007). This is a process that involves intensive chemistry and hazardous reagents, unlike biotransformation with *B. bassiana*. The expensive cost of AHC restricts experimental trials and research design with this substrate. Once the catalytic activity of *B. bassiana* is enhanced with n-alkanes and DHEA as substrate, those parameters may be applied for the biotransformation of AHC.

Table 1: Cost of related substrates and products of interest.

Substrate	Cost ($/mg)	Product	Cost ($/mg)
DHEA	0.0096	-	-
(A) 7-alpha-hydroxy-4-cholesten-3-one	500	7-alpha-12 alpha-dihydroxy-4-cholesten-3-one	3,150
(B) 11-deoxycortisol	14	Cortisol	0.02
(C) Deoxycorticosterone	0.23	Corticosterone	0.13

Culture Media

Challenges to the application of fungal catalysts lie in consistent induction of oxidative enzymes for specific biocatalytic reactions (Iida 1998 and Dolfing 2003). Efforts in culture medium development for growth and biotransformation have advanced the field, but the catalytic inventory of *B. bassiana* is not well characterized.

In our lab, *B. bassiana* was adapted to n-C_{12} and n-C_{16} as sole carbon source to induce oxidative gene expression. The induction process for expressing Cytochrome P-450 is defined as adapting cells to n-alkanes in the culture medium. Strain ATCC 7159 has been transferred to a minimal medium containing solely n-alkanes as carbon source at 15-day intervals. The adaptation process requires a repetitive method every 15 days. After starting growth of *B. bassiana* on n-alkane, after 15 days, a 10% inoculum from the previous culture was grown on a new fresh n-alkane medium. This procedure has been repeated since summer 2011. After inducing oxidative P-450 enzymes, the biotransformation of DHEA was performed in a Corn Steep Liquor-glycerol culture medium. Glycerol is an important component in the epicuticle of insects. Fats in the epicuticle are composed of a glycerol head group and three fatty acid tails. The enzymes

involved in the degradation of these hydrocarbons reduce these lipids, and release the glycerol molecule for further degradation as carbon source.

HPLC

The analysis of product distribution in a sample by means of High Performance Liquid Chromatography (HPLC) was performed. HPLC parameters and retention time of a molecule are associated with its chemical and physical arrangement and are useful in the characterization of steroid mixtures with efficiency and ease (Synovec 1984). HPLC is commonly applied to the separations of steroids and is a top technique for identifying components in a mixture. HPLC analysis was performed following the protocol of Xiong et al. (2006) using a Shimadzu instrument. HPLC is a valuable instrument used for characterization since there is a correlation between the chemical structures of a metabolite to its retention time in the separation column. Elution order may appear to be "backwards", because for HPLC on C-18 columns, items are separated by polarity and hydrophobic absorption occurs. For this reason, standards from pure compounds were used to validate retention times of each metabolite. HPLC was used to track and identify steroid production during the time course of experiments. Substrate conversion and yields of reactions were quantified with HPLC results and initial concentration of substrate.

Evaluation Parameters

To quantify the performance of the process, parameters like substrate conversion, selectivity, and yield of desired product were evaluated. The conversion of DHEA was calculated by evaluating the maximum of "conversion % vs. time of reaction" plot. The

amount (mM) of identified metabolites was used to calculate product yield. The selectivity was calculated using the known amounts of desired hydroxy-product and byproducts. These equations defined below, are the criteria used to evaluate *B. bassiana* performance as a biocatalyst. For sample calculations, see Appendix A. For molecular weights of substrate, desired product and by product, see Table A1.

$$Conversion_{DHEA} = \frac{Moles_{\ Reacted}}{Moles_{Initials}}$$

Equation 1: Conversion of substrate to products.

$$Selectivity_{11a-OH\ Product} = \frac{Moles_{\ 11a-OH\ Product}}{Moles_{Byproduct}}$$

Equation 2: Biocatalyst selectivity of desired products.

$$Yield_{11a-OH\ Product} = \frac{Moles_{\ 11a-OH\ Experimental}}{Moles_{11a-OH\ Theoretical}}$$

Equation 3: Reaction yield of desired products.

Summary

B. bassiana is a significant biocatalyst that can be optimized. The central hypothesis is to enhance the biocatalyst potential by mimicking an epicuticle environment with hydrocarbons. Cells will express oxidative cytochrome P-450 enzymes with the induction of n-alkanes, and these enzymes will hydroxylate unfunctionalized carbons during the conversion of steroids to more valuable compounds. To evaluate this hypothesis a model steroid, DHEA was chosen. The next Chapter will show how bioconversion of the compound DHEA was enhanced by n-alkane induction.

CHAPTER 2
N-ALKANE SOLVENT-ENHANCED BIOTRANSFORMATION OF STEROID DHEA BY *BEAUVERIA BASSIANA* AS BIOCATALYST

Introduction

Previous studies confirmed that in the presence of n-alkane solvents, such as n-dodecane (n-C_{12}) and n-hexadecane (n-C_{16}), *B. bassiana* increases the gene expression of cytochrome-P450 monooxygenase enzymes (Pedrini 2007 and 2010). Since this class of enzymes plays an important role in the conversion of xenobiotic compounds to valuable chemical intermediates, the objective of the work presented in this Chapter was to achieve higher hydroxylation capacity using n-alkanes, which have been shown to enhance biopesticide efficacy. To prove that these enhancements can impact steroid biotransformations, an alkane-induction system was designed for selective hydroxylation. The advantage of using fungal biotransformation is that this strategy provides cost effective and eco-friendly production of steroids that cannot be easily synthesized by solely chemical means. Hence, compounds that require a great amount of effort to synthesize can be more easily produced with the use of microbial transformations.

Our interest was to engineer a biocatalytic system that enhances the hydroxylation of unfunctionalized carbons during the conversion of steroids to more valuable metabolites. As a result, portions of this Chapter were published in the article: "N-alkane Solvent-enhanced Biotransformation of Steroid DHEA by *Beauveria bassiana* as Biocatalyst" *Journal of Advances in Biology & Biotechnology* 2(1): 30-37, 2015; Article no. JABB.2015.005. The catalytic activity of *B. bassiana* was screened using

dehydroepiandrosterone (DHEA) as substrate. Previous studies showed that the hydroxylated derivatives of DHEA serve as metabolic intermediates in the biosynthesis of ring-D lactones through Baeyer–Villiger oxidation. In addition, literature reports on steroid transformation by *B. bassiana* provide examples of regioselective α-hydroxylation in C-7, C-19 and C-21 on steroids. Our work has shown that the selectivity in the conversion of DHEA with strain ATCC 7159 differed from biotransformations performed with other strains reported in the literature, which claim to obtain the activation of an unfunctionalized carbon to 11α-hydroxy-17-oxo derivatives before obtaining a 3β,11α,17β-triol product. The results of our current studies provide a potentially new method to synthesize innovative, biologically functioning steroids.

Experimental

Materials

n-Alkane solvents and salts were purchased from Fisher Scientific. House deionized water was further purified for experimental work using a Thermo Fisher Barnstead Nanopure Ultrapure™ water purification system. DHEA, PDB and Corn Steep liquor were purchased from Sigma Aldrich. Intermediate androstenediol was purchased from Steraloids (Newport, RI).

Microorganism

Beauveria bassiana was purchased from the American Type and Culture Collection (ATCC). This strain, ATCC 7159, was isolated from a lab contaminant and

routinely grown on potato dextrose broth (PDB).

Preparation of n-alkanes medium (NM)

n-Alkanes (NM) medium was prepared by mixing 0.4 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.6 g $MgSO_4.7H_2O$, 1.0 g KCl and 0.7 g of $NH_4NO_3.7H_2O$ per liter of distilled water. The pH was adjusted to 5 with either HCl or NaOH. The medium was distributed in the appropriate Erlenmeyer fermentation flasks covered with a foam stopper/aluminum foil and sterilized by autoclave. Before inoculation, the medium was supplemented with synthetic hydrocarbons (10% v/v) as follows: 50 mL of n-C_{12} and 50 mL of n-C_{16}.

Preparation of glycerol - corn steep liquor medium (GM)

Glycerol-corn steep liquor medium (GM) was prepared by mixing 20 g of corn steep liquor and 10 g of glycerol per liter of water. The pH of the brown suspension was adjusted to 7 with either HCl or NaOH. The medium was distributed in the appropriate Erlenmeyer fermentation flasks covered with a foam stopper/aluminum foil and sterilized by autoclave and allowed to cool before inoculation.

Preparation of buffer solution

The buffer solution was prepared by mixing 2.09 g $Na(NH_4)HPO_4 \cdot 4H_2O$ and 1.74 g K_2HPO_4 per liter of distilled water. The pH was adjusted to 7 with either HCl or NaOH. The buffer solution was distributed into Erlenmeyer fermentation flasks covered with a foam stopper/aluminum foil, and sterilized by autoclave. The solution was allowed to cool down and 5 mL of a sterile dextrose (2M) solution was added. The dextrose was

sterilized by passing it through 0.45 µm filter during the addition to the buffer.

Biotransformation with resting cells

Inoculum growth on n-alkanes: Cells were harvested (10% v/v) from Potato Dextrose Broth (PDB) directly into NM (Phase 1). These cells were grown and adapted to n-C_{12} and n-C_{16} for 15 days at 250 RPM and 26°C. Another 10% v/v inoculum from this culture was used to repeat the process and inoculate a new NM. This repetitive process increases oxidative gene expression and targets the degradation of hydrocarbons. Multiple generations (49) were grown successfully using n-alkanes as carbon source from June 2011 to September 2013.

Conditions of cultivation and transformation: Phase 1 cultures (40 mL inoculum) were washed twice with sterile water and transferred to 400 mL of GM, contained in a 1L Erlenmeyer flask (Phase 2). The flask was incubated at 250 RPM and 26°C for 3 days. The fermented medium was centrifuged at 1,956 G for 10 minutes, washed with Buffer (3 x 50 mL) and re-suspended in 200 mL of buffer, contained in a 1L Erlenmeyer flask (Phase 3). DHEA dissolved in ethanol was added over Phase 3 resting cells. The flask was incubated at 250 RPM and 26°C for the reaction time indicated.

Biotransformation with control (Cells not exposed to n-alkanes): Cells were harvested (10% v/v) from Potato Dextrose Broth (PDB) directly into GM. These cells were grown for 15 days at 250 RPM and 26°C. A 40 mL inoculum from the previous solution was washed twice with sterile water and transferred to 400 mL of GM, contained

in a 1L Erlenmeyer flask. The flask was incubated at 250 RPM and 26°C for 3 days. The fermented medium was centrifuged at 1,956 G for 10 minutes, washed with Buffer (3 x 50 mL) and re-suspended in 200 mL of buffer, contained in a 1L Erlenmeyer flask. DHEA dissolved in ethanol was added and the flask was incubated at 250 RPM and 26°C for the reaction time indicated.

Biotransformation with intermediate (androstenediol): Phase 1 cultures (40 mL inoculum) were washed twice with sterile water and transferred to 400 mL of GM, contained in a 1L Erlenmeyer flask. This flask was incubated at 250 RPM and 26°C for 3 days. The fermented medium was centrifuged at 1,956 G for 10 minutes, washed with Buffer (3 x 50 mL) and re-suspended in 200 mL of buffer, contained in a 1L Erlenmeyer flask. Androstenediol dissolved in ethanol was added over the resting cells. The flask was incubated at 250 RPM and 26°C for the reaction time indicated.

Isolation and identification of products

After transformation, the reaction liquid was centrifuged and steroids were extracted from the supernatant with ethyl acetate (3 x 50 mL). The pellet of cells was discarded. After removal of the solvent, metabolites were analyzed with thin layer chromatography (TLC) and separated by silica gel column chromatography (2 cm × 10 cm) eluting with a gradient of chloroform/methanol (12:1, v/v). Compounds were visualized by spraying TLC plates with a solution of phosphomolybdic acid (1:10, v/v) and heating at 105°C until color developed. High performance liquid chromatography (HPLC) was performed with a Shimadzu instrument equipped with a C18 column (4.6

mm × 125 mm). Isocratic column elution was monitored by a photodiode array detector. The wavelength was set at 250 nm and a methanol–water (60:40, v/v) mobile phase was eluted at a flow rate 0.5 mL/min. The ^{1}H NMR Spectra were recorded at room temperature on a 300 MHz Bruker Avance spectrometer using deuterated chloroform ($CDCl_3$) as a solvent and tetramethylsilane as an internal standard.

Results

Products isolated in the course of DHEA transformation

After 7 days of transformation of 200 mg of DHEA dissolved in 5 mL of ethanol with cells adapted to n-alkanes, 65 ± 6.3 % of the substrate was metabolized. Results are means ± Std Dev., n = 3. Figure 8 shows the structure of the biotransformation products. Isolated products included (average mg ± standard deviation, mol %): androstenediol (**I**) (81.2 ± 12.7 mg, 40.3%) and 3β,11α,17β-trihydroxyandrost-5-ene (**II**) (48.6 ± 6.9 mg, 22.8%). In experiments with cells that were not adapted to n-alkanes, 17% of the substrate was metabolized. Isolated products in this case included (mol %): **I** (23.8 ± 3.4 mg, 11.8%) and **II** (10.2 ± 6.3 mg, 4.78%). Experiments were performed in triplicates.

Table 2: Isolated products (mol %) after 7 days of biotransformation with cells adapted and non-adapted to n-alkanes. Results are means ± Std Dev., n = 3.

Carbon source:	Conversion (%)	Selectivity of 11α-OH (%)	Yield of 11α-OH (%)
n-alkane induced	65 ± 3	60 ± 6	39 ± 3
No-alkane	17 ± 3	41 ± 4	7.4 ± 2

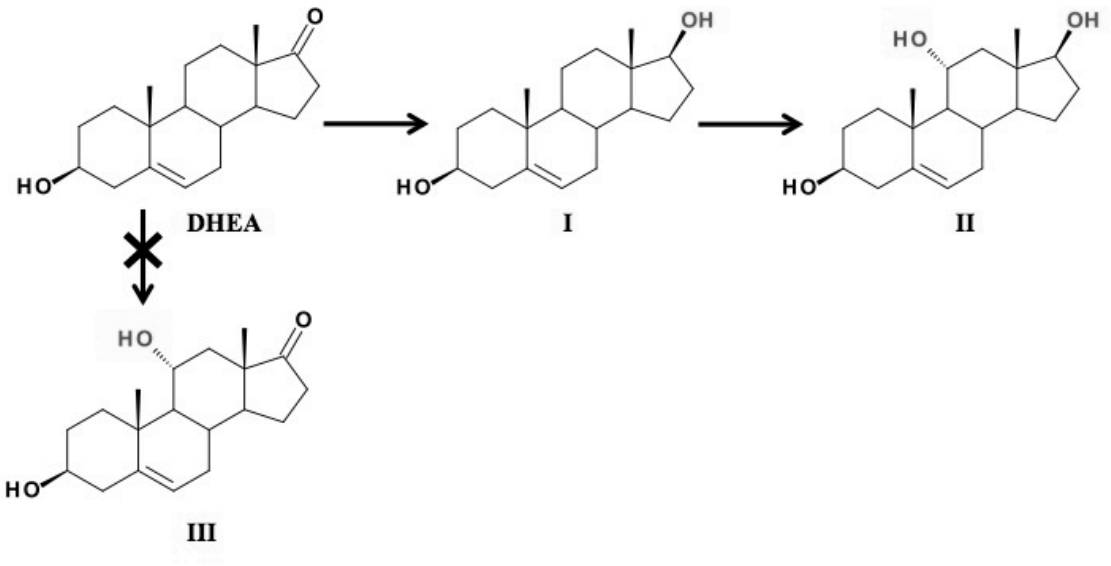


Figure 8: DHEA to 11α-hydroxylation pathway. After 7 days DHEA biotransformation, there was isolated (mol %): androstenediol (**I**) (40.3%) and 3β,11α,17β-trihydroxyandrost-5-ene (**II**) (22.8%). No 11α-Hydroxy-DHEA (**III**) was synthesized.

Structural identification of metabolites

Incubation of DHEA with *B. bassiana* ATCC 7159 gave two metabolites that were separated by chromatography on silica. The first metabolite was identified as androstenediol (**I**) and was identified by comparison of its NMR data to that of DHEA. ^{1}H NMR spectrum of **I** had a new resonance signal at δH 3.64 ppm (t) consistent with the addition of a proton during the reduction of C-17 ketone. The main metabolite, 3β,11α,17β-trihydroxyandrost-5-ene (**II**) was identified by comparison of its NMR data to that of compound **I**. ^{1}H NMR spectrum of **II** had a new resonance signal at δH 3.93 ppm (dt) consistent with substitution at an equatorial proton. Downfield β-carbon shifts in the ^{13}C NMR spectra for C-9 (Δ 7.9 ppm) and C-12 (Δ 18.4 ppm) confirmed hydroxylation at C-11 (See Table 3.). Details of the NMR analysis are as follows:

Androstenediol (**I**) GCMS 290.22 m/z (lit. 290.44). ^{1}H NMR ($CDCl_3$) δ_H: 0.88 (3H, s, 18-H), 1.02 (3H, s, 19-H), 3.54 (1H, m, 3α-H), 3.67 (1H, t, J = 8.4 Hz, 17α-H), 5.35 (1H, d, J = 5.1 Hz, 6-H). *Rf* in ethyl acetate/chloroform (3:7): 0.65; *Rt:* 5.23 min.

Table 3: ^{13}C NMR data for starting material DHEA and metabolites androstenediol (**I**) and 3β,11α,17β-trihydroxyandrost-5-ene (**II**) determined in $CDCl_3$. Downfield β-carbon shifts for C-9 and C-12 confirmed hydroxylation at C-11.

Carbon atom	Compound		
	DHEA	**I**	**II**
1	39.8	39.9	43.3
2	34.1	34.1	36.5
3	74.1	74.4	75.5
4	44.8	44.9	46.5
5	143.7	143.5	145.8
6	123.4	124.0	125.1
7	34.1	34.1	35.3
8	34.0	34.6	35.8
9	52.8	52.9	60.8
10	39.2	39.2	42.4
11	23.0	23.3	72.6
12	33.4	39.4	57.8
13	50.1	45.4	47.4
14	54.4	54.0	54.7
15	24.5	26.1	27.2
16	38.4	33.2	33.7
17	223.9	84.5	85.1
18	16.1	13.6	15.5
19	22.0	22.1	22.4

3β,11α,17β-trihydroxyandrost-5-ene (**II**) GCMS306.22 m/z (lit. 306.43). ^{1}H NMR ($CDCl_3$) δ_H: 0.81 (3H, s, 18-H), 1.21 (3H, s, 19-H), 3.55 (1H, m, 3α- H), 3.63 (1H, t, J=8.5 Hz, 17α-H), 3.93 (1H, dt, J=4.5 Hz, J=12.0 Hz, 11β-H), 5.33 (1H, d, J = 5 Hz, 6-

H). *Rf* in ethyl acetate/chloroform (3:7): 0.44; HPLC *Rt:* 8.31 min.

Determination of bioconversion pathway in the hydroxylation reaction

In order to investigate pathways of DHEA transformation with n-alkane induced cells, the composition of mixtures sampled after various transformation periods was studied. The data for the time course of experiments are shown in Table 4. The analysis indicates that the first stage of the process was the reduction of the C-17 ketone in DHEA. The resulting 3,17-hydroxy derivative was further metabolized through α-hydroxylation (to 11α-hydroxy); these derivatives did not appear to be further metabolized to other compounds. In all cases of transformations, the 3,17-hydroxy derivatives were detected earlier than 11α-hydroxy products.

Table 4. Composition of crude mixtures obtained in transformations of DHEA by *Beauveria bassiana* determined by HPLC analysis (%).

Metabolite	HPLC	Time of transformation (day)						
	R_t (min)	1	2	3	4	5	6	7
Reaction with cells grown with n-alkanes								
DHEA	2.96	78	58	43	37	36	37	35
Androstenediol (**I**)	5.23	22	42	55	53	47	39	41
3β,11α,17β-trihydroxyandrost-5-ene (**II**)	8.31	0	0	2	10	17	24	24
Reaction without n-alkanes								
DHEA	2.97	95	90	86	85	83	84	83
Androstenediol (**I**)	5.25	5	10	14	13	14	12	12
3β,11α,17β-trihydroxyandrost-5-ene (**II**)	8.32	0	0	0	2	3	4	5

In order to confirm the notion that only 3,17-hydroxy steroids undergo the 11α-hydroxylation, an experiment was carried out, in which intermediate (**I**) was added as a substrate to the *B. bassiana* culture induced by n-alkanes (rationale: to double check the

affinity of α-hydroxylase enzymes to different sites). The reaction mixture after 7 days of transformation of 200 mg of substrate (**I**) contained 49 mg (0.24 mg/mL) of 11α-hydroxy derivative (**II**) and 144 mg (0.72 mg/mL) of substrate (**I**) (See Figure 9.). No other metabolites were detected besides unreacted (**I**) and newly synthesized (**II**), at a similar amount produced in previous DHEA biotransformations (48 mg of **II**), thus confirming that all intermediate **I** goes to desired steroid **II**.

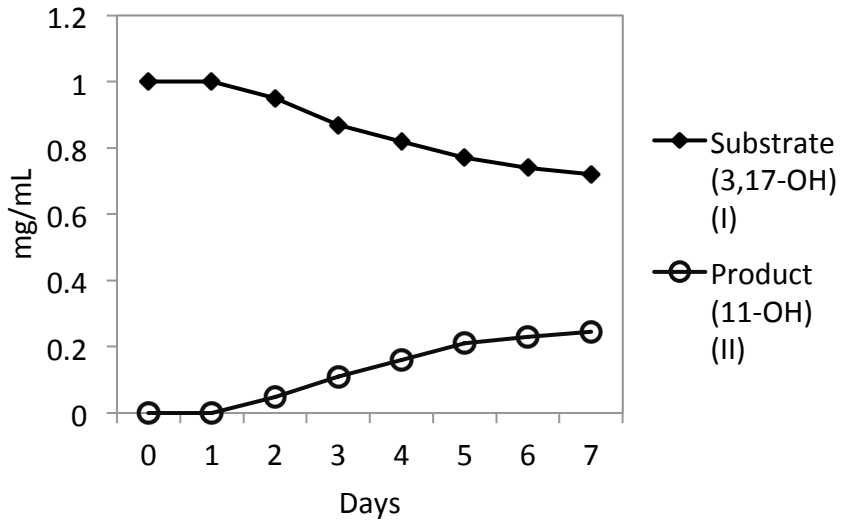


Figure 9: The intermediate androstenediol (**I**) was reacted as a substrate, separate from DHEA, to confirm the notion that only 3,17-hydroxy steroids undergo the 11α-hydroxylation. The reaction mixture after the transformation of (**I**) contained 0.24 mg/mL of 11α-hydroxy derivative (**II**) and 0.72 mg/mL of substrate (**I**). No new metabolites were detected (**II**).

Results indicated that enzymes catalyzing the hydroxylation reaction are inducible. In experiments with cells adapted to n-alkanes, the reaction mixture, after 3 days of incubation with DHEA contained 55% of **I**, and after another 4 days, it decreased to 41%. The 11α-hydroxy derivative **II** grew from 2 to 24% in 4 days. In examining results with cells never exposed to n-alkanes, the reaction mixture, after 3 days of

incubation with DHEA, contained 14% of **I.** After another 3 days, the amount of **I** decreased to 12%. The 11α-hydroxy derivative **II** grew from 2 to 5% in 3 days. The amount of 3,17-hydroxy and 11α-hydroxy derivatives in the reaction mixtures, sampled at the same time of incubation with cells adapted to n-alkanes was higher. This suggests that n-alkanes induced the enzymes catalyzing the reduction of C-17 ketone and the 11α-hydroxylation of DHEA

Discussion

In the culture of the strain *B. bassiana* ATCC 7159, the 3-hydroxy-17-oxo was transformed in a one-step process to a 3,17-dihydroxy steroid. Analysis of composition of the product mixtures as a function of reaction time indicates that only the 3,17-dihydroxy derivatives of DHEA undergo the 11α-hydroxylation. After 4 days transformation, the reaction mixture contained 3,17-dihydroxy metabolite **I** together with 11α-hydroxy **II**, while other by-products were not detected. 11α-hydroxy-DHEA was not identified in any of the reactions, which suggests that the reduction of the C-17 ketone of DHEA is preceded by the 11α-hydroxylation. This differs from reports in the literature, which suggested the activation of an unfunctionalized carbon to 11α-hydroxy-17-oxo **III** derivatives before obtaining a 3β,11α,17β-triol product (Swizdor 2011).

The observed differences of catalytic activity of *B. bassiana* ATCC 7159 results from growing the cells with n-alkane solvents as carbon source. Comparison of the metabolism of DHEA with cells adapted and non-adapted to n-alkanes, indicates that the

n-alkane solvents are active inducers of Steroid-11α-hydroxylase (See Figure 10.). The selectivity of 11α-hydroxy (ratio of **II** and 3-hydroxy-17-oxo derivatives **I)** increased from 41% to 60%, and the cultures produced 19.27 mg more of desired product, just by growing the inoculum of cells with n-alkanes as the carbon source.

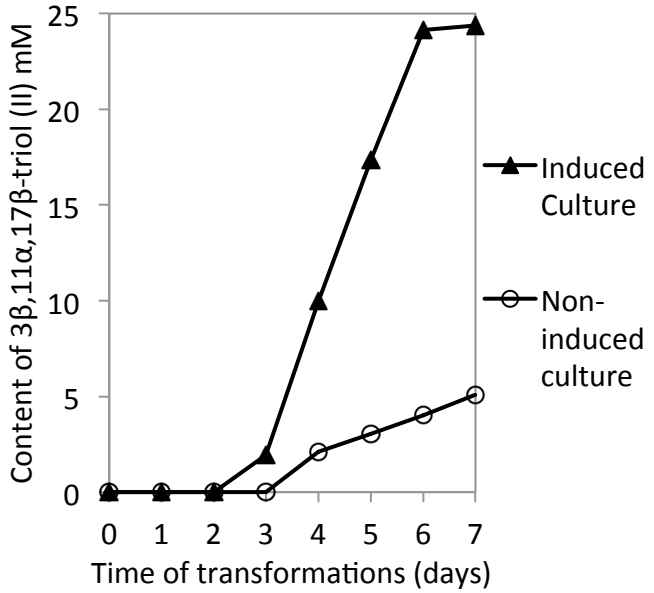


Figure 10: Comparison of content of 3β,11α,17β-trihydroxyandrost-5-ene (**II**) in the mixtures after transformation of DHEA by the non-induced and n-alkane-induced cultures of *Beauveria bassiana*.

It seems that the reduction of the ketone to 17β-alcohol is critical for controlling the amount of resulting 11α-hydroxy **II**. The amount of 17β-alcohol in the mixture after 4 days did not exceed 53%. During transformation by the induced cells, the amount of 3β,11α,17β-triol **II** reached 24% (19% more than non-induced cells). The use of n-alkanes as growth substrates implicates several continuous transformations and involves an intensive gene expression of enzymes like alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthase, to provide the desired fatty acyl-CoA for the β-

oxidation metabolic pathway (Gotoh 1993 and Holder 2007). Cytochrome-P450 monooxygenase enzymes of the strain ATCC 7159 are distinguished from others described in the literature, which catalyze hydroxylation of C-11, by the fact that they oxidize substrates with a 17β alcohol group. To confirm the specific participation of P-450's in the biotransformation, future work on protein and gene expression is needed. With these results, *B. bassiana* ATCC 7159 appears as a promising fungus that could be used on industrial processes to enhance the synthesis of hydroxylated steroids via a biohydroxylation process.

Conclusions

These results show that the synthesis of 11α-hydroxylated steroids is enhanced when DHEA is subjected to biotransformation when *B. bassiana* has been adapted to n-alkanes as carbon source. The appearance of the products suggests that the reduction of the C-17 ketone of DHEA precedes the 11α-hydroxylation reaction when n-alkanes are present. This differs from reports in the literature, which proposed the activation of an unfunctionalized carbon to 11α-hydroxy-17-oxo derivatives (**III**) before obtaining a 3β,11α,17β-triol product **II**. The next Chapter shows the kinetics of the reaction, and identifies the reaction conditions that improve the hydroxylation of unfunctionalized carbons during the conversion of DHEA.

CHAPTER 3
OPTIMIZATION OF THE 11A-HYDROXYLATION OF STEROID DHEA BY SOLVENT-ENHANCED *BEAUVERIA BASSIANA*

Introduction

To extend the use of *B. bassiana* to commercial application, optimization of reaction conditions and accurate prediction of biotransformation products are necessary. One area where fungal transformations have been actively applied is in bioconversion of steroids, which represent one of the largest sectors in pharmaceutical industry with an annual market value over $10 billion (Morgan B.P. 2000 and Faria 2007). In our research, *B. bassiana* was applied in selective oxidations for the synthesis of steroids to increase the application as a practical biocatalyst and to further improve oxidative selectivity. The rationale for a successful biotransformation arises from the potential induction of oxidative enzymes in environments, which mimic natural conditions for *B. bassiana.*

When *B. bassiana* was cultivated in the presence of n-hexadecane (n-C_{16}), the gene expression of eight cytochrome (CYP) P450 monooxygenase enzymes was presumably induced (Pedrini 2010). These enzymes have a tremendous potential in drug development (Kumar 2010). In Chapter 2, CYP's potential was targeted with the design of a biotransformation process involving n-C_{16} for the possible induction of CYP genes (Gonzalez 2015). In this Chapter, the process was optimized for selective oxidation and eco-friendly production of 11α-hydroxy steroid derivatives that cannot be easily synthesized by solely chemical methods (Pfeifer 1989 and Hanson 2003). As a result,

portions of this Chapter were submitted to the article: "Optimization of the 11α-Hydroxylation of Steroid DHEA by Solvent-Enhanced *Beauveria bassiana*" in the *Journal of Advances in Biocatalysis and Biotransformation*.

Our interest is to characterize the system and identify reaction conditions that improve the hydroxylation of unfunctionalized carbons during the preparation of steroids. The catalytic activity of *B. bassiana* was studied with dehydroepiandrosterone (DHEA). Previous studies showed that the reaction pathway of DHEA derivatives varies with biocatalytic strain and substrate and reaction parameters. Furthermore, low yields of valuable 11α-hydroxy intermediates are currently obtained (Xiong 2006). Our work establishes the reaction sequence of DHEA to hydroxylated intermediates with strain ATCC 7159, and defines the reactor arrangement, kinetics, substrate concentration, temperature, and pH for optimal production of these steroids.

Experimental

Microorganism and Materials

Beauveria bassiana was purchased from the American Type and Culture Collection (ATCC). This strain, ATCC 7159, was isolated from a lab contaminant and routinely grown on potato dextrose broth (PDB). n-Alkane solvents and salts were purchased from Fisher Scientific. House deionized water was further purified for experimental work using a Thermo Fisher Barnstead Nanopure Ultrapure water purification system. DHEA, PDB and Corn Steep liquor were purchased from Sigma

Aldrich. Androstenediol was purchased from Steraloids (Newport, RI).

Media preparation

The n-alkane medium (NM) was prepared by mixing 0.4 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.6 g $MgSO_4.7H_2O$, 1.0 g KCl and 0.7 g $NH_4NO_3.7H_2O$ per liter of distilled water, at pH 5. pH was adjusted with HCl. After sterilization, the medium was supplemented with n-hexadecane (n-C_{16}) (10% v/v). Glycerol medium (GM) was prepared by mixing 20 g of corn steep liquor and 12.6 mL of glycerol per liter of water, at pH 7. Buffer solution was prepared by mixing 2.09 g $Na(NH_4)HPO_4 \cdot 4H_2O$ and 1.74 g K_2HPO_4 per liter of distilled water, at pH 7. When the solution was at room temperature, 5 mL of 2M dextrose was added. The dextrose was sterilized by passing it through 0.45 µm filter during buffer addition.

Inoculum growth on n-alkanes

Cells were harvested (10% v/v) from Potato Dextrose Broth (PDB) directly into NM (Phase 1). These cells were grown and adapted to n-C_{16} for 15 days at 250 RPM and 26°C. Another 10% v/v inoculum from this culture was used to repeat the process and inoculate a new NM. Multiple generations (61) were grown successfully from June 2011 to April 2014 using n-alkanes as the carbon source.

Biotransformation procedure with Resting Cells

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to 200 mL of GM. The flask was incubated at 250 RPM and 26°C for 3 days.

This culture was centrifuged at 1,956 G for 10 mins; cells were washed with buffer (3 x 50 mL) and re-suspended in 200 mL of buffer. A DHEA solution (1 mL) was added to resting cells to give a 200 mg/mL starting concentration. The flask was incubated at 250 RPM and 26°C for 7 days.

Biotransformation procedure with Growing Cells

A slant of *B. bassiana* ATCC-7159, kept in potato dextrose agar, was transferred to 25 mL of GM. The flask was incubated at 250 rpm and 28°C for 3 days. This culture was transferred into 200 mL of GM in a 500 mL Erlenmeyer flask. The flask was incubated at 250 RPM and 28°C for 1 day. A DHEA solution (1 mL) was added to this culture to give a 200 mg/mL starting concentration. The flask was incubated at 250 RPM and 26°C for 7 days.

Biotransformation procedure with different Volume Ratio

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred into flasks containing 25 mL, 50 mL, 75 mL and 100 mL of GM. These flasks were incubated at 250 RPM and 26°C for 3 days. These cultures were centrifuged at 1,956 G for 10 mins. Cells were washed with buffer (3 x 50 mL) and re-suspended in 25 mL of buffer. DHEA (25 mg) dissolved in ethanol was added to each flask and incubated at 250 RPM and 26°C for 7 days.

Biotransformation procedure for Biocatalyst Saturation Effect

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to 7 flasks containing 100 mL of GM. These flasks were incubated at 250 RPM and 26°C for 3 days. These cultures were centrifuged at 1,956 G for 10 mins. Cells were washed with buffer (3 x 50 mL) and re-suspended in 100 mL buffer. A DHEA solution (1 mL) was added to resting cells to produce flasks with the following starting concentrations: 0.25 mg/mL, 0.52 mg/mL, 0.83 mg/mL, 1 mg/mL, 1.33 mg/mL, 2 mg/mL, 2.5 mg/mL. Flasks were incubated at 250 RPM and 26°C for 7 days.

Biotransformation procedure for Kinetics of Intermediate Reaction

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to 4 flasks containing 100 mL of GM. These flasks were incubated at 250 RPM and 26°C for 3 days. These cultures were centrifuged at 1,956 G for 10 mins. Cells were washed with buffer (3 x 50 mL) and re-suspended in 100 mL buffer. An androstenediol solution (1 mL) was added to resting cells to produce flasks with the following starting concentrations: 0.20 mg/mL, 0.45 mg/mL, 0.60 mg/mL, 0.75 mg/mL. Flasks were incubated at 250 RPM and 26°C for 7 days.

Biotransformation procedure for Temperature-pH Effect

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to flasks containing 25 mL of GM. These flasks were incubated at 250 RPM and 26°C for 3 days. These glycerol-grown cultures were centrifuged at 1,956 G for 10 mins. Cells were washed with buffer (3 x 50 mL) and re-suspended in 25 mL of buffer.

DHEA dissolved in ethanol (25 mg/mL initial concentration) was added to each flask, and the pH for buffer solutions in each flask was 5, 6, 7, 8 and 9. For each flask, pH was adjusted with HCl or NaOH. Five flasks for each pH value were incubated separately at 15°C, 20°C, 26°C, 30°C, and 35°C at 250 RPM for 7 days.

Biotransformation with Different Agitation Rates

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to five flasks containing 25 mL of GM. These flasks were incubated at 250 RPM and 26°C for 3 days. These cultures were centrifuged at 1,956 G for 10 mins. Cells were washed with buffer (3 x 50 mL) and re-suspended in 25 mL of buffer. 25 mg/mL of DHEA dissolved in ethanol was added to each flask and incubated at 100, 200, 250, 300 and 400 RPM respectively, at 26°C for 7 days.

Isolation and identification of products

After biotransformation, the buffer was centrifuged at 1,956 G for 10 mins, and steroids were extracted from the liquid supernatant with ethyl acetate (3 x 50 mL). The pellet of cells was discarded. After removal of the solvent, metabolites were analyzed with thin layer chromatography (TLC) and separated by silica gel column chromatography (2 cm × 10 cm) eluting with a gradient of chloroform/methanol (12:1, v/v). Compounds were visualized by spraying TLC plates with a solution of phosphomolybdic acid (1:10, v/v) and heating at 105°C until color developed. High performance liquid chromatography (HPLC) was performed with a Shimadzu instrument equipped with a C18 column (4.6 mm × 125 mm). Isocratic column elution was

monitored by a photodiode array detector. The wavelength was set at 250 nm, and a methanol–water (60:40, v/v) mobile phase was eluted at a flow rate 0.5 mL/min. The ^{1}H NMR spectra were obtained at room temperature on a 300 MHz Bruker Avance spectrometer using deuterated chloroform ($CDCl_3$) as a solvent and tetramethylsilane as an internal standard.

Results

Structural identification of metabolites

Incubation of DHEA with *B. bassiana* ATCC 7159 gave two metabolites that were separated by chromatography on silica (See Figure 11.). The identification of these steroids followed the protocol of Chapter 2, Results section, "Structural identification of metabolites" subsection. The first metabolite was identified as androstenediol (**I**) and the second metabolite, 3β,11α,17β-trihydroxyandrost-5-ene (**II**). ^{1}H NMR spectrum of **II** had a new resonance signal at δH 3.93 ppm (dt) consistent with substitution at an equatorial proton. Downfield β-carbon shifts in the ^{13}C NMR spectra for C-9 (Δ 7.9 ppm) and C-12 (Δ 18.4 ppm) confirmed hydroxylation at C-11 (See Figure 12.).

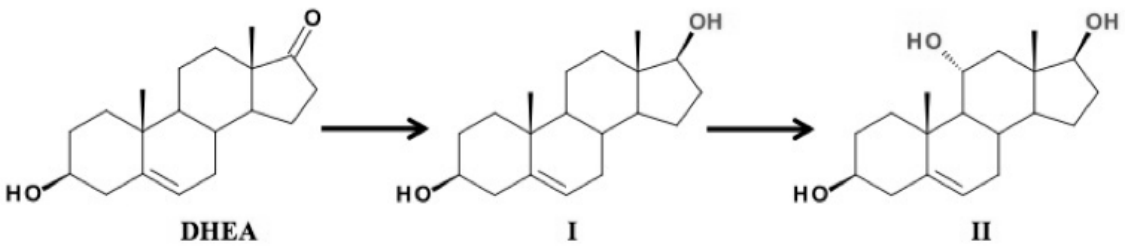


Figure 11: The 11α-hydroxylation of DHEA with *B. bassiana* leads to androstenediol (**I**) and 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

Androstenediol (**I**): GCMS 290.22 m/z (lit. 290.44). ^{1}H NMR ($CDCl_3$) δ_H: 0.88 (3H, s, 18-H), 1.02 (3H, s, 19-H), 3.54 (1H, m, 3α-H), 3.67 (1H, t, J = 8.4 Hz, 17α-H), 5.35 (1H, d, J = 5.1 Hz, 6-H). ^{13}C NMR (CDCl3): δ (ppm): 143.5 (C-5), 124.0 (C-6), 84.5 (C-17), 74.4 (C-3), 54.0 (C-14), 52.9 (C-9), 45.4 (C-13), 44.9 (C-4), 39.9 (C-1), 39.4 (C-12), 39.2 (C-10), 34.6 (C-8), 34.1 (C-7), 34.1 (C-2), 33.2 (C-16), 26.1 (C-15), 23.3 (C-11), 22.1 (C-19), 13.6 (C-18). *Rf* in ethyl acetate/chloroform (3:7): 0.65; HPLC *Rt:* 5.23 min.

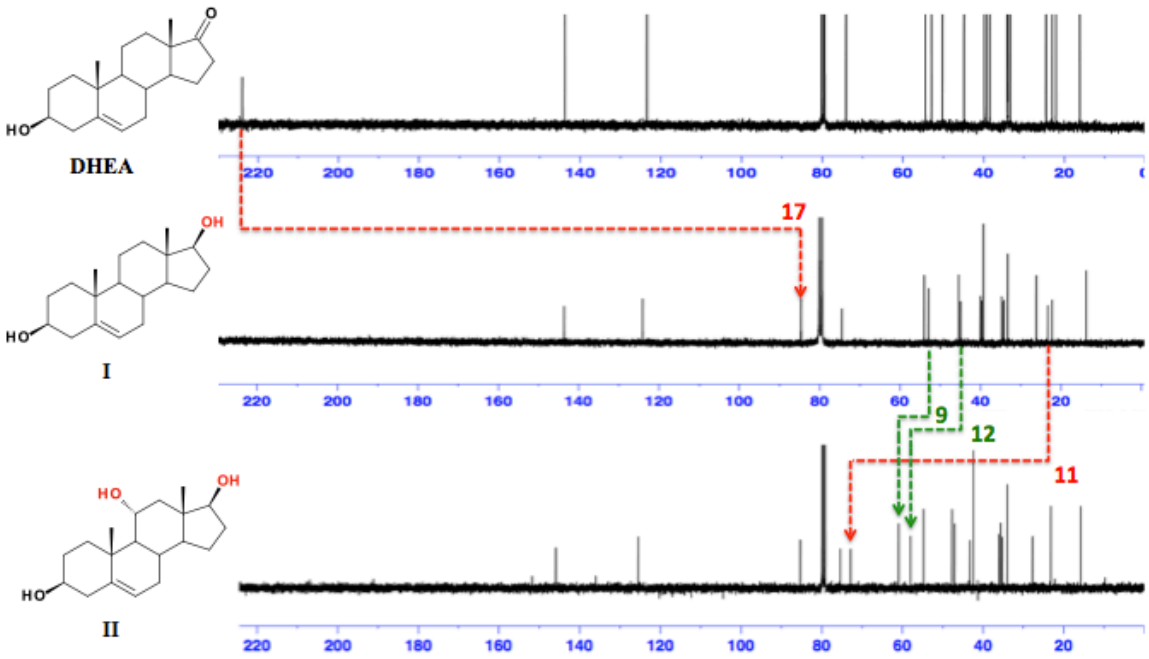


Figure 12: Correlation of ^{13}C chemical shifts for DHEA, androstenediol (**I**) and 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

3β,11α,17β-trihydroxyandrost-5-ene (**II**): GCMS 306.22 m/z (lit. 306.43). ^{1}H NMR ($CDCl_3$) δ_H: 0.81 (3H, s, 18-H), 1.21 (3H, s, 19-H), 3.55 (1H, m, 3α- H), 3.63 (1H, t, J=8.5 Hz, 17α-H), 3.93 (1H, dt, J=4.5 Hz, J=12.0 Hz, 11β-H), 5.33 (1H, d, J = 5 Hz, 6-

H). ^{13}C NMR (CDCl3): δ (ppm): 145.8 (C-5), 125.1 (C-6), 85.1 (C-17), 75.5 (C-3), 72.6 (C-11), 60.8 (C-9), 57.8 (C-12), 54.7 (C-14), 47.4 (C-13), 46.5 (C-4), 43.3 (C-1), 42.4 (C-10), 36.5 (C-2), 35.8 (C-8), 35.3 (C-7), 33.7 (C-16), 27.2 (C-15), 22.4 (C-19), 15.5 (C-18). *Rf* in ethyl acetate/chloroform (3:7): 0.44; HPLC *Rt:* 8.31 min.

Determination of the reaction sequence

To establish the pathway of DHEA derivatives, the composition of mixtures sampled after various transformation periods was analyzed. Results indicate that the first stage in the reaction was the reduction of the C-17 ketone of DHEA. The resulting 3,17-hydroxy derivative was further metabolized through 11α-hydroxylation. These derivatives did not appear to be further metabolized to other compounds. In all cases of transformations, the 3,17-hydroxy derivative was detected before the 11α-hydroxy product.

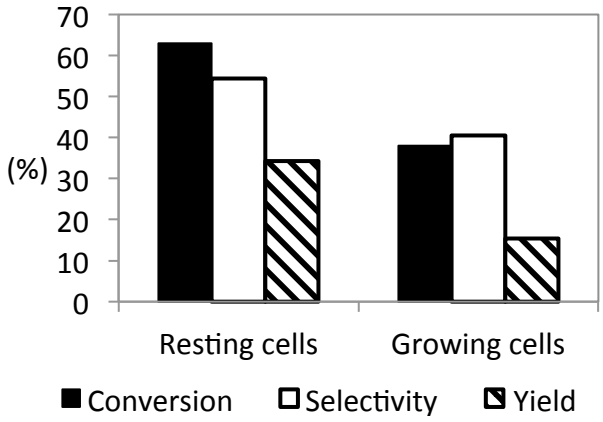


Figure 13: Higher amount of steroids were synthesized with "Resting cells", and the selectivity and yield of desired 11α-hydroxy **II** increased as well.

Biotransformation with Resting and Growing Cells

After 7-day biotransformations of 200 mg of DHEA, experiments with resting cells resulted in 63% substrate conversion (See Figure 13.). Isolated products included: 66 mg of androstenediol (**I**) and 38 mg of 3β,11α,17β-trihydroxyandrost-5-ene (**II**). The selectivity and yield of desired 11α-hydroxy **II** product were 54.3% and 34.2% respectively. In experiments with growing cells, 38% of the substrate was metabolized. Isolated products included: 53.2 mg of **I** and 22.8 mg **II**. The selectivity and yield of desired 11α-hydroxy **II** product were 40.4% and 15.3% respectively.

Biotransformation with different Volume Ratio

Table 5 shows the results from 7-day biotransformations of 25 mg of DHEA in 25 mL of resting cells with different GM to buffer solution ratio (See Table 1**)**. Figure 14 shows the effect of "volume ratio" in the yield of desired product **II** from the experiments in Table 5. Experiments were performed in triplicates.

Table 5: Biotransformations results of 25 mg of DHEA with different volumes of growth medium GM and buffer solution. Results are means ± Std Dev., n = 3.

Cells grown on glycerol GM (mL)	Re-suspended in Buffer (mL)	Ratio	DHEA Conversion (%)	I (mg)	II (mg)	Selectivity of II (%)
25	25	**1**	54.6 ± 3.3	8.1 ± 0.9	5.7 ± 0.6	65.7 ± 3.7
50	25	**2**	67 ± 4.0	9.7 ± 1.0	7.0 ± 0.7	67.8 ± 2.8
75	25	**3**	71.0 ± 5.0	10.2 ± 1.2	7.5 ± 0.9	68.9 ± 3.3
100	25	**4**	72.0 ± 5.0	10.2 ± 1.4	7.7 ± 1.0	69.6 ± 5.7

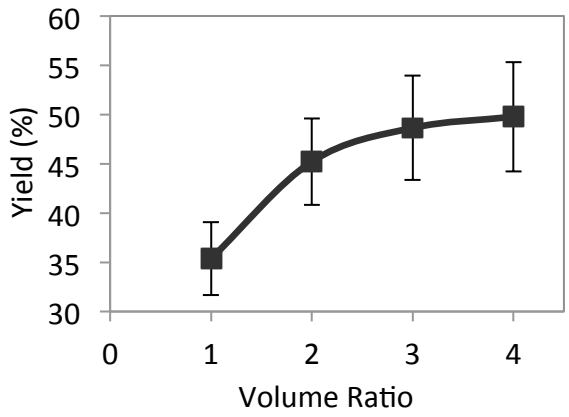


Figure 14: Effect of high volumes of glycerol medium GM compared to buffer solution (higher ratios) in biotransformations. Results are means ± Std Dev., n = 3.

Biotransformation for Biocatalyst Saturation Effect

Figures 15 and 16 show the results of 7-day biotransformations with different concentrations of DHEA in 100 mL of resting cells. Values for reaction rates, conversion, selectivity and yield were determined for each condition. Experiments were performed in triplicates. For sample calculations of reaction rate see Appendix A.

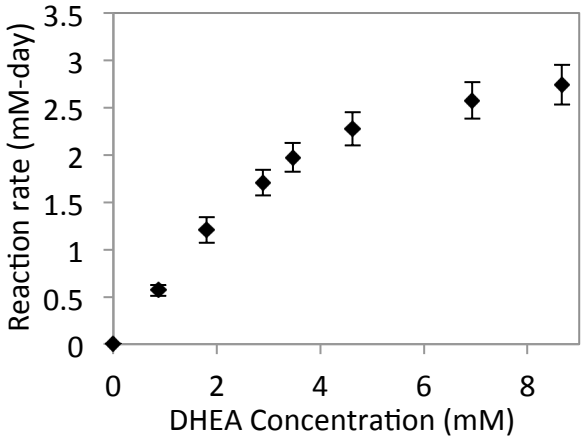


Figure 15: Effect of initial substrate concentration on reaction rate. As DHEA concentration increases, the rate of reaction goes up at a directly proportional rate until *B. bassiana* saturates. Results are means ± Std Dev., n = 3.

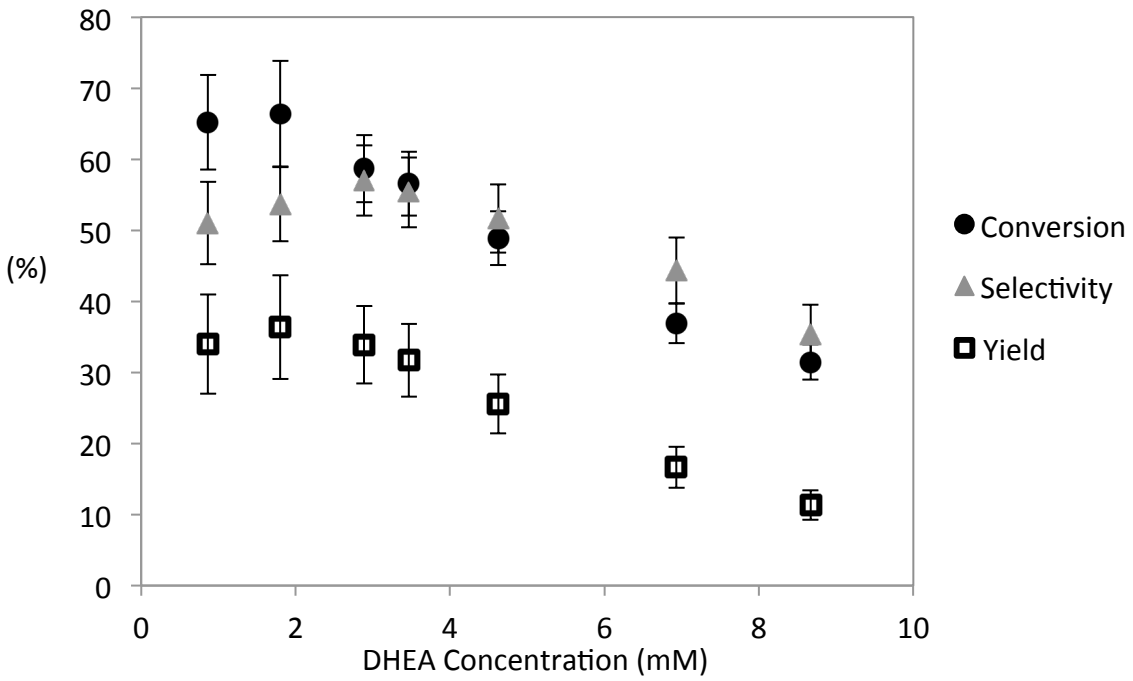


Figure 16: Effect of initial substrate concentration in the conversion of DHEA, selectivity and yield of desired product **II**. Results are means ± Std Dev., n = 3.

Biotransformation for Kinetics of Intermediate Reaction

We extend the validity of the quasi-steady state assumption for reactions with intermediate androstenediol (P_1) and pseudo first-order kinetics, to calculate specific reaction parameters and determine the limiting kinetic step in the overall reaction. A well-known mathematical method for dealing with a system of ordinary differential equations in two-step reactions is the quasi-steady state assumption (QSSA) (Frenzen 1988). This analytical solution was used to compare our results with numerical solutions of the differential equations describing our reaction. Experimental results permitted the application of the quasi-steady state assumption to the reaction:

$$S \xrightarrow{k_1} P_1 \xrightarrow{k_2} P_2$$

The differential equations describing these reactions are:

$$\frac{d[S]}{dt} = -k_1\,[S]$$

Equation 4: Differential equation describing the time dependent change in substrate concentration, [S].

$$\frac{d[P_1]}{dt} = k_1\,[S] - k_2\,[P_1]$$

Equation5: Differential equation describing the time dependent change in intermediate concentration, [P_1].

$$\frac{d[P_2]}{dt} = k_2\,[P_1]$$

Equation 6: Differential equation describing the time dependent change in final product concentration, [P_2].

The "overall reaction rate" υ is the rate of formation of $P_1 + P_2$, thus:

$$\upsilon_{overall} = \frac{d[P_{Total}]}{dt} = \frac{d[P_1]}{dt} + \frac{d[P_2]}{dt} = V_1 + V_2$$

Equation 7: The "overall reaction rate" υ is the rate of formation of $P_1 + P_2$, in terms of differential equations.

Adding equations 5 and 6 into equation 7:

$$\upsilon_{overall} = \frac{d[P_{Total}]}{dt} = k_1\,[S] - k_2\,[P_1] + k_2\,[P_1]$$

Equation 8: Adding equations 5 and 6 into equation 7 results in the rate of production in terms of reaction constants.

$$\upsilon = \frac{d[P_2]}{dt} = \frac{V_{max}\,[S]}{K_M + [S]}$$

Equation 9: Michaelis-Menten kinetics describes the rate of reaction (υ) from DHEA to 3β,11α,17β-trihydroxyandrost-5-ene (**II**). Here, V_{Max} represents the overall maximum rate

achieved by *B. bassiana* at saturating substrate concentrations. The overall Michaelis Constant K_M is concentration of DHEA at which the reaction rate is half V_{max}.

Rewriting equation 8 and 9 in terms of overall Michaelis-Menten kinetics constants:

$$\upsilon_{overall} = \frac{V_{max}\,[S]}{K_M + [S]} = k_1\,[S]$$

Equation 10: Rewriting equation 8 and 9 in terms of overall kinetics constants, only when the 1st reaction is running at *Vmax*.

Assuming that at high substrate concentration, K_M is negligible ([S] >>> K_M):

$$\upsilon_{overall} = \frac{V_{max}\,[S]}{[S]} = V_{max}$$

Equation 11: Assuming that at high substrate concentration ([S] >>> K_M), therefore the velocity of a reaction is equal to its maximum velocity.

Km and Vmax for the second reaction cannot be determined because the dependence of rate on substrate concentration is linear (See Figure L2). However, the slope of Figure L2 is *Vmax*/Km, with units of day^{-1} (per day). Since overall values V_{max} and K_M were previously calculated, equation 10 was used to calculate k_1 at a known [S].. Solving equation 10 for k_1 resulted in 0.53 day^{-1} (See Appendix A for sample calculations). Using the rapid equilibrium approximation, we assumed that the enzyme-substrate complex equilibrate very rapidly, therefore the instantaneous velocity is the catalytic rate that is equal to the maximum velocity (See equation 11). We assumed that at high substrate concentration ([S] >>> K_M), the overall reaction rate ($\upsilon_{overall}$) was equal to its V_{max} (4.45 mM/day).

Biotransformation for Temperature-pH Effect

Figure 17 shows the results of 7-days biotransformations of 25 mg of DHEA in 25 mL of resting cells with temperatures and pH between 15°C - 35°C and 5-9, respectively. Values for conversion, yield and selectivity were determined for each condition.

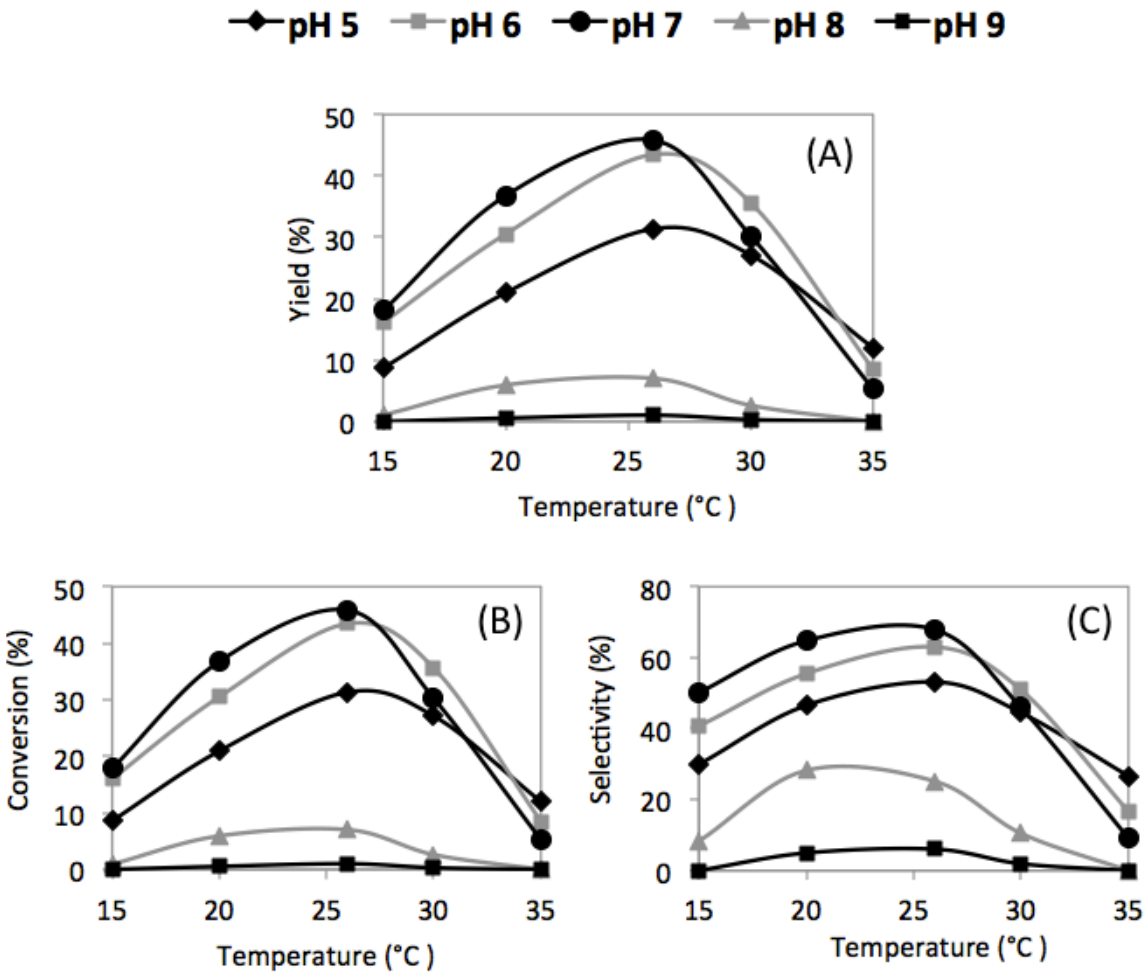


Figure 17: Effect of temperature and pH on DHEA conversion (B), selectivity (C) and yield of 11α-hydroxy steroid **II** (A).

Biotransformation with Different Agitation Rates

Figure 18 shows the results of 7-day biotransformations with different agitation rates between 100 and 400 RPM. Values for conversion and yield were determined for each condition.

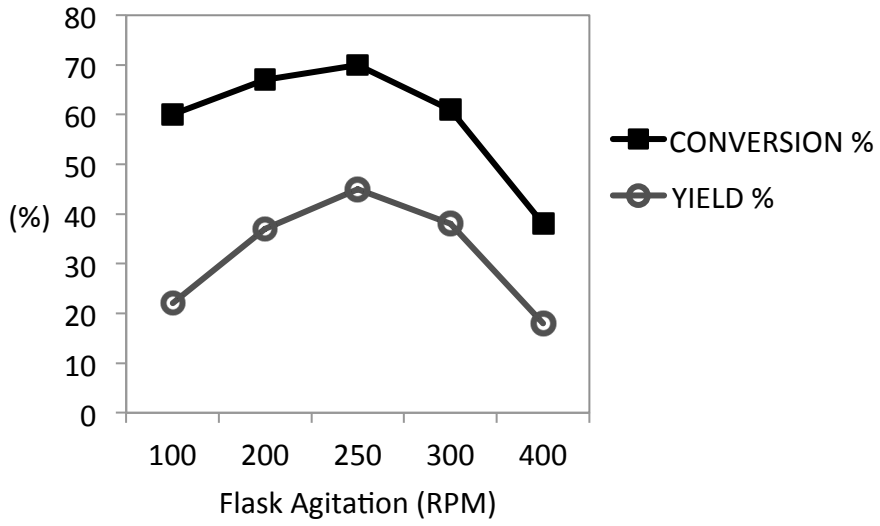


Figure 18: Effect of flask agitation on conversion of DHEA and production yield of 11α-hydroxy steroid **II**.

Discussion

B. bassiana strain ATCC 7159 transformed 3-hydroxy-17-oxo DHEA to 3,17-hydroxy steroid **I**. Analysis of composition of the product mixtures as a function of reaction time indicates that **I** undergoes an 11α-hydroxylation to synthesize 3,11,17-trihydroxy **II** in all reactions, as shown in Chapter 2. Other by-products were not detected.

The biotransformation of DHEA was carried out under different reaction conditions using arrangements of "growing cells" and "resting cells" (See figure 19.). Biotransformations with “growing cells” are commonly used because of the simplicity of time, enzyme recycle and process arrangement. Experiments with "resting cells" involve

an extra step of isolation from nutrients and resuspension of cells in a buffer. When 1.0 mg/mL of DHEA was subject to both configurations, 63% of the substrate was converted to products with "resting cells" and 38% with "growing cells". In addition, higher amount of steroids **I** and **II** were synthesized with "resting cells", and the selectivity and yield of desired 11α-hydroxy **II** was increased as well (See Figure 13.). The observed differences of conversion might be attributed to the competitive inhibition between substrate and components of the medium in "growing cells". In this environment, enzymes may preferentially catalyze the medium instead of reacting with the substrate (Kim 2002). This limitation can be overcome by increasing initial DHEA concentrations since the presence of an inhibitor can be overcome by high substrate concentrations (Reed 2010). In "resting cells", the buffer in which the biotransformation occurs contains a very dilute concentration of nutrients that cannot compete with the concentration of substrate.

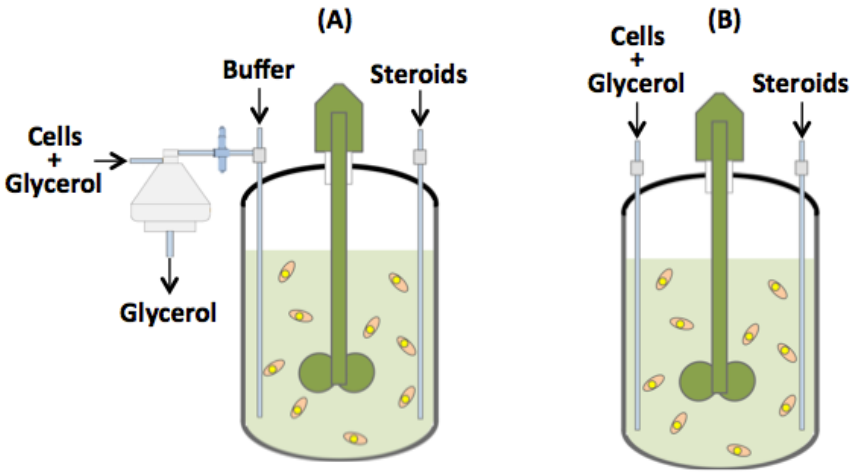


Figure 19: Reactor arrangements; (A) Resting cells at left, are suspended in a buffer and isolated from nutrients. This arrangement resulted in a more efficient reaction. (B) Growing cells at right are constantly growing and performing the biotransformation at the same time. This arrangement resulted in a poor yield reaction.

Experiments in resting cells with different media volume ratios between **GM** commonly used for cell growth and buffer solution used for reaction, revealed the optimum ratio for DHEA conversion. In most cases, a ratio of 1 is used, but our research explored the effects of a higher ratio in efficacy and product distribution. Results showed that higher volumes of **GM** compared to buffer (higher ratios) increase the amount of DHEA derivatives, conversion, selectivity and desired product yield. It seems that higher volume ratios increase the value of these parameters to a similar shape of a negative exponential curve, which suggests the saturation or inhibition of the proteins responsible for the reaction (See Figure 14.). The biotransformation of 1.0 mg/mL with volume ratio 1 and 4 resulted in 65 and 69% selectivity of desired **II** respectively. A similar value in selectivity (between 65-69%) was observed for all experiments. This means that increasing the amount of cells and biomass with volume ratio, will not affect reaction selectivity. Interestingly, there was a large enhancement in the conversion of DHEA from 54.6 to 72.0%. There was also a remarkable increment in the yield of desired product II from 35.3 to 49.7%. This improvement might be explained by assuming that the expression of CYP's responsible for the biotransformation increases proportionally to the amount of biomass produced with **GM**. Higher volume ratios mean dense concentration of cells expressing CYP proteins for the biotransformation of DHEA.

The rate of reaction in the biotransformation processes greatly depends on the initial substrate concentration (Cook 2014). Substrate conversion, selectivity of the fungus and the yield of desired product are also sensitive to initial DHEA concentration. To better understand these parameters, experiments were performed with different initial

concentrations of DHEA ranging from 0.25 mg/mL (0.86 mM) up to 2.5 mg/mL (8.6 mM). The apparent rate of reaction observed fits a Michaelis-Menten kinetics model (See equation 10) (Nakamura 2002). The rate of reaction increases constantly with higher concentrations of DHEA (See Figure 20), and the overall maximum reaction rate (V_{max}) was 4.45 mM/day with a K_M of 4.86 mM; calculated with a nonlinear regression. As DHEA concentration increases, the rate of reaction goes up at a directly proportional rate until the reaction becomes saturated with the substrate at V_{max}. This fact can be explained by assuming that at saturation point, the reaction will not speed up and adding extra substrate will make no difference (Thomas 2002). Interestingly, quite the opposite is observed in the conversion, selectivity and efficiency of the reactions (See Figure 16.). Using a diluted amount of substrate (0.52 mg/mL) results in a desired product yield of 36.3%, which is higher than the 11.3% yield obtained at high concentrations of DHEA (2.5 mg/mL). This presumably results from the interaction of large amount of biocatalyst

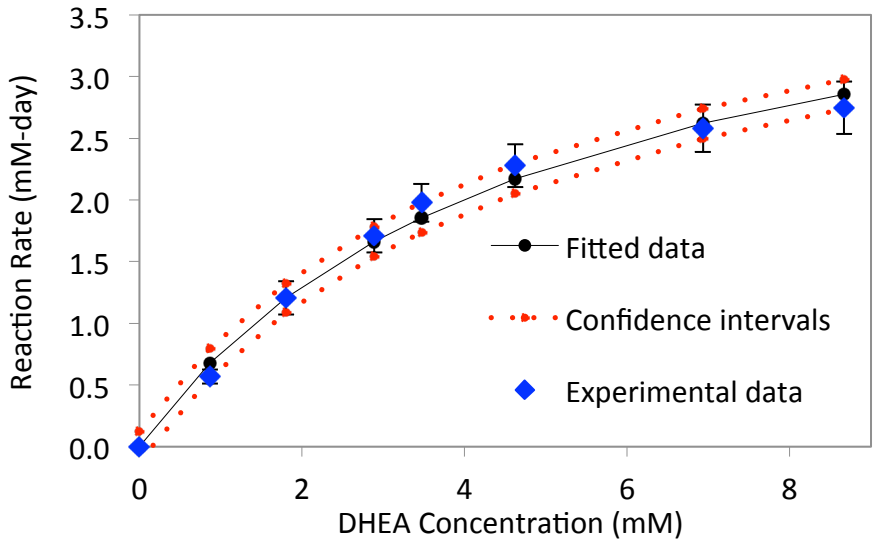


Figure 20: Apparent reaction parameters were established by fitting reaction rate results with a non-linear regression using Michaelis-Menten kinetics from Equation 9.

relative to a small concentration of substrate. In overall, if the initial concentration of substrate is chosen properly at a mid range (0.83-1.0 mg/mL), considerable rate of reaction, high conversion, selectivity and yield, could be achieved.

The overall reaction rate (4.45 mM/day) is limited by the rate of formation of intermediate product androstenediol (**I**). To better understand this, the rates of reaction for both steps in the production of 3β,11α,17β-trihydroxyandrost-5-ene (**II**) were calculated. The rate of reaction of DHEA to androstenediol calculated with a non-linear regression is 2.37 mM/day, and the rate of reaction of androstenediol to 3β,11α,17β-trihydroxyandrost-5-ene calculated with Equation 7 is 2.08 mM/day (See example of calculation in Appendix A Part 5). Since V_1 is higher than V_2, the limiting step (slowest) in the reaction is the transformation of intermediate androstenediol (**I**) to desired 3β,11α,17β-trihydroxyandrost-5-ene (**II**) (See Table 6.).

Table 6: Michaelis-Menten kinetics parameters for the two-step reaction. Results showed that the rate-limiting step in the process is the second reaction (androstenediol (**I**) to desired 3β,11α,17β-trihydroxyandrost-5-ene (**II**)).

	Substrate DHEA to intermediate **I**	Intermediate **I** to desired product **II**	Overall reaction
K_M (mM)	2.77	-	4.86
V_{MAX} (mM/d)	2.37	2.08	4.45

It has been shown that pH and temperature of a reaction greatly affect *B. bassiana* efficiency and product distribution (Xiong 2006). Experiments showed that if the initial pH value ranges between 6.0 and 7.0, high conversion (70%), yield (45.8%) and selectivity (67.8%) could be achieved at mid temperatures (20-30°C). At pH 5, the

conversion of DHEA was similar (61%) to pH 7 and the yield of desired steroid **II** decreased considerably (31%). At high pH 8 and 9, low conversion of substrate was obtained (17-28%) and very low yield of **II** (1-7%). Poor yield was also obtained in experiments with low (15-20°C) and high temperatures (35°C). Interestingly, changes in temperature and pH do not induce the synthesis of new steroids besides **I** and **II**, but have a considerable effect on the efficiency of *B. bassiana*. It seems that 26°C and pH range 6-7 are the best values to synthesize desired steroid **II** (See Figure 6.). This fact can be explained by assuming that these parameters have an essential effect on CYP activity at mild environments (Daniel 2010).

The aeration rate of *B. bassiana* during the biotransformation was found crucial for the biocatalyst performance. The aeration rate for Erlenmeyer flasks is correlated to the agitation of the flask in the incubator. In these experiments, the change in conversion and yield of desired product was constant through the different agitation rates (RPM). Experiments showed that at low and high RPM (100 and 400 respectively), a low yield (18-22%) of II is achieved. The conversion of DHEA obtained at these RPM values was the lowest (37.9%). At RPM values of 200 and 300, a considerable conversion of substrate (65%) and yield of II (35%) was achieved, but it was shown that the optimum agitation rate for the biotransformation of DHEA was 250 RPM. At this value, the maximum conversion and yield were achieved (70 and 45% respectively). Interestingly, the changes of agitation rate have a considerable effect on the efficiency of *B. bassiana*. This fact can be explained by assuming that at low RPM (100), the agitation is not sufficient to provide the necessary aeration for *B. bassiana* to thrive and react at its

maximum capacity. Also, low agitation rates may be insufficient to adequately suspend the microcrystalline substrate in the reaction medium. As well, at high RPM (400), the vigorous agitation can cause stress and cell damage, which decreases the efficiency of the biocatalyst.

Conclusions

B. bassiana may have very useful prospects in the pharmaceutical industry because it can lead to high yields of valuable 11α-hydroxy intermediates. The optimization of the reaction parameters can facilitate significant advancement in the application of *B. bassiana* on multiple reactions of commercial interest (Hou 2014). Two products were observed when DHEA is subject to biotransformation with *B. bassiana* strain ATCC 7159 under different reactor configurations, volume of growth medium, substrate concentration, temperature and pH of reaction. Desired steroid **II** was synthesized more efficiently in a resting cell arrangement with a GM/Buffer ratio of 4, with moderate DHEA concentration (0.83-1.0 mg/mL), and a mild environment (26°C, pH 7). The limiting step (slowest) in the reaction was determined to be the transformation of intermediate androstenediol (**I**) to desired 3β,11α,17β-trihydroxyandrost-5-ene (**II**). These results show the ability of *B. bassiana* to adapt and metabolize a substrate in different biotransformation settings. More importantly, these results suggest that this fungus presents a promising future as biocatalyst to be used for enhancement in the production of drug metabolites. The next Chapter shows the relations between cell growth conditions and accurate prediction of biotransformation products.

CHAPTER 4
CELL GROWTH CORRELATIONS WITH THE ENHANCEMENT OF THE BIOTRANSFORMATION OF DHEA WITH *BEAUVERIA BASSIANA*

Introduction

The complex regulation of cellular functions in the area of pest control is well known, but the relation between cell growth and oxidative biocatalysis has not been fully explored (Boron 2nd ed., Gaby 1996 and Gao 2011,). In Chapter 2, we confirmed that the use of n-hexadecane (n-C_{16}) during cell growth, enhanced the oxidative capacity of *B. bassiana,* and in Chapter 3 we established the optimal reaction parameters and kinetics of the oxidative reaction. In this Chapter, we established experimental correlations between cell growth conditions and accurate prediction of biotransformation products. This Chapter describes, (1) cell growth rate with n-alkanes and glycerol medium respectively, and (2) the relation between biomass production with substrate conversion, rate of reaction, and yield of hydroxylated products.

These parameters define a well-characterized system and provide the opportunity to optimize the production process for selective biooxidations of commercial interest. To prove that these enhancements can impact steroid biotransformations, experiments were designed with different number of cells and composition of culture media. Design of experiments (DOE) was performed with QI Macros software to understand the effect of each experimental parameter as a variable factor. The objective of the work presented in this Chapter was to achieve a *higher* reaction rate, and *increase* the hydroxylation capacity and biomass production using n-alkanes as carbon source. Moreover, we are

looking to enhance the efficacy of biopesticide application practices to improve biocatalytic oxidations.

Experimental

Media preparation

The n-alkane medium (NM) was prepared by mixing 0.4 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.6 g $MgSO_4.7H_2O$, 1.0 g KCl and 0.7 g $NH_4NO_3.7H_2O$ per liter of distilled water, at pH 5. pH was adjusted with HCl. After sterilization, the medium was supplemented with n-hexadecane (n-C_{16}) (10% v/v) (Crespo et al. 2000). Glycerol medium (GM) was prepared by mixing 20 g of corn steep liquor and 12.6 mL of glycerol per liter of water, at pH 7. Buffer solution was prepared by mixing 2.09 g $Na(NH_4)HPO_4{\cdot}4H_2O$ and 1.74 g K_2HPO_4 per liter of distilled water, at pH 7. When the solution was at room temperature, 5 mL of 2 M dextrose was added. The dextrose was sterilized by passing it through 0.45 µm filter during the addition to the buffer.

Inoculum growth on n-alkanes

Cells were harvested (10% v/v) from Potato Dextrose Broth (PDB) directly into NM (Phase 1). These cells were grown and adapted to n-C_{16} for 15 days at 250 RPM and 26°C. Another 10% v/v inoculum from this culture was used to repeat the process and inoculate a new NM. Multiple generations (61) were grown successfully from June 2011 to April 2014 using n-alkanes as the carbon source.

Measurements of Wet Biomass produced with cells adapted to n-alkanes

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to 400 mL of GM. Flasks were incubated at 250 RPM and 26°C for 13 hours (During exponential phase). Samples of 25 mL were collected every 2, 5, 8, 11 and 13 hrs. A small portion of the sample (1 mL) was used to measure the absorbance at 600 nm with a Beckman Du640 spectrophotometer. The rest of the sample (24 mL) was vacuum filtered, and the biomass was collected on a weighing sheet and weighed. Experiments were performed in triplicates.

Characterization of Cell Growth

Characterizing growth curve of control (Cells neither exposed nor adapted to n-alkanes): Cells were harvested (10% v/v) from Potato Dextrose Broth (PDB) directly into GM. These cells were grown for 15 days at 250 RPM and 26°C. A 40 mL inoculum from the previous solution was washed twice with sterile water and transferred to 400 mL GM. Flasks were incubated at 250 RPM and 26°C for 37 hours. Samples of 1 mL were extracted from the flask every hour and analyzed on a Beckman Du640 spectrophotometer at 600 nm absorbance. Experiments were performed in triplicates.

Characterizing growth of cells adapted to n-alkanes: Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to 400 mL GM. Flasks were incubated at 250 RPM and 26°C for 37 hours. Samples of 1 mL were extracted from the flask every hour and analyzed on a Beckman Du640 spectrophotometer at 600nm absorbance. Experiments were performed in triplicates.

Biotransformation procedure with different Biomass Concentrations

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to 200 mL GM. The flask was incubated at 250 RPM and 26°C. The time of incubation was determined with the amount of biomass to be used for reaction. This was calculated using the calibration/growth curve of wet biomass previously developed (See Figure 1.). Reactions were performed with concentrations of biomass (mg/mL): 3.04, 4.83, 6.75, 8.51 and 10.46. When the flask reached the corresponding biomass concentration, the culture was centrifuged at 1,956 G for 10 mins. Cells were washed with buffer (3 x 50 mL) and re-suspended in 200 mL buffer. An ethanolic solution of DHEA (1 mL)) was added to the resting cells to give an initial concentration of 200 mg/mL. Each flask was incubated at 250 RPM and 26°C for 7 days.

Biotransformation procedure for Rate of Reaction Effect with different Biomass Concentrations and substrate concentrations

Phase 1 cultures (10% v/v inoculum) were washed twice with sterile water and transferred to flasks containing 100mL of GM. These flasks were incubated at 250 RPM and 26°C. The time of incubation was determined based on the amount of biomass to be used for reaction. Target concentrations were determined using the calibration/growth curve of wet biomass previously developed (See Figure 21.). Reactions were performed with high, medium, and low concentrations of biomass (mg/mL): 3.04 (low biomass), 6.10 (medium biomass), and 10.46 (high biomass). When the flasks reached the desired biomass concentration, the cultures were centrifuged at 1,956 G for 10 mins. Cells were washed with buffer (3 x 50 mL) and re-suspended in 100 mL buffer. DHEA (1 mL of a

200 mg/mL ethanolic solution) was added to each flask. Flasks were incubated at 250 RPM and 26°C for 7 days. The initial DHEA concentrations for low biomass were 0.3, 0.75, 1.25, 1.75, 2.15 and 2.5 mg/mL. The DHEA concentrations for medium biomass were 0.35, 0.76, 1.3, 1.8, 2.25 and 2.5 mg/mL. The DHEA concentrations for high biomass were 0.25, 0.52, 1, 1.5, 2 and 2.5 mg/mL.

Isolation and identification of products

After biotransformation, steroids were extracted, separated and analyzed with High performance liquid chromatography, following the protocol described in Chapters 2 and 3: "Isolation and identification of products".

Results

Wet biomass and Growth Curves

Figure 21 shows the results of the average wet biomass produced with cells adapted to n-alkanes in glycerol medium during the exponential growth phase. The natural log of the biomass was plotted to fit a linear equation with 0.973 R^2 (See Figure 21.). When cells adapted to n-alkanes were used to inoculate a GM; the growth rate at the exponential phase between 4 and 13 hours was 0.58 hr^{-1}, and the maximum optical density at the stationary phase was 8.21 (See Figure 22.). Sample calculations are presented in appendix A. When control cells non-adapted to n-alkanes were used to inoculate a GM; the growth rate at the exponential phase between 6 and 24 hours was 0.23 hr^{-1}, and the maximum optical density at the stationary phase was 6.57.

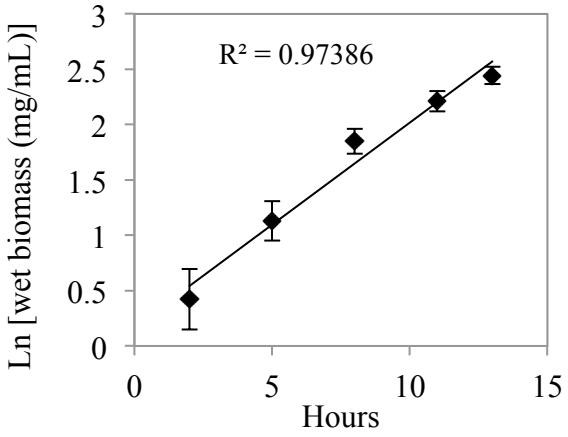


Figure 21: Rate of wet biomass produced during the exponential growth phase of strain ATCC 7159 previously adapted to n-hexadecane.

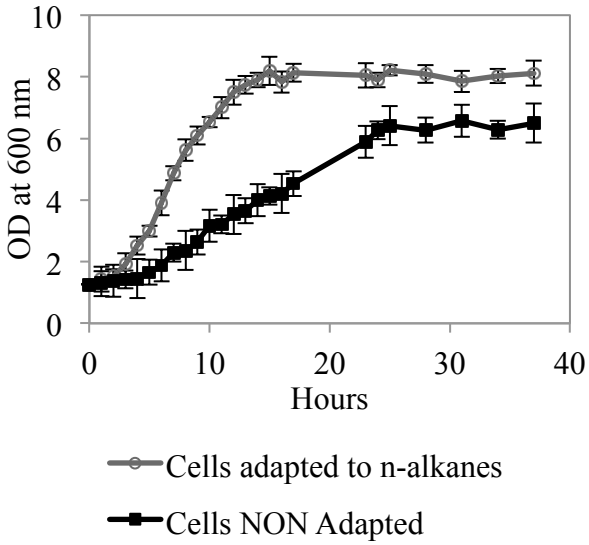


Figure 22: Growth curves of cells adapted and non-adapted to n-hexadecane, shows a difference in growth rate of cells and the maximum optical density reached. Samples were diluted 1/10 with water after an OD of 1.5. The OD observed was multiplied by 10, to calculate the real OD of the sample. Results are means ± Std Dev., n = 3.

Structural identification of metabolites

The bioconversion pathway of DHEA resulted in the same reaction mechanism as described in Chapters 2 and 3 (See Figure 11.). Incubation of DHEA with *B. bassiana* ATCC 7159 gave two metabolites that were separated by chromatography on silica,

following the protocol in Chapter 2: "Structural identification of metabolites". The first metabolite was identified as androstenediol (**I**) by comparison of its NMR data to that of DHEA. The second metabolite, 3β,11α,17β-trihydroxyandrost-5-ene (**II**) was identified by comparison of its NMR data to that of compound **I**.

Biotransformation with different Biomass Concentrations

Figures 23 and 24 show the results of 7-day biotransformations with different biomass concentrations in 100 mL of resting cells. Values for conversion, selectivity and yield were determined for each condition. The substrate concentration falls smoothly inversely proportional to the amount of cells used, and reactions carried out with high biomass reach a plateau after 4 days with a small decrease in substrate remaining after 6 days (maximum substrate conversion) (See Figure 23.).

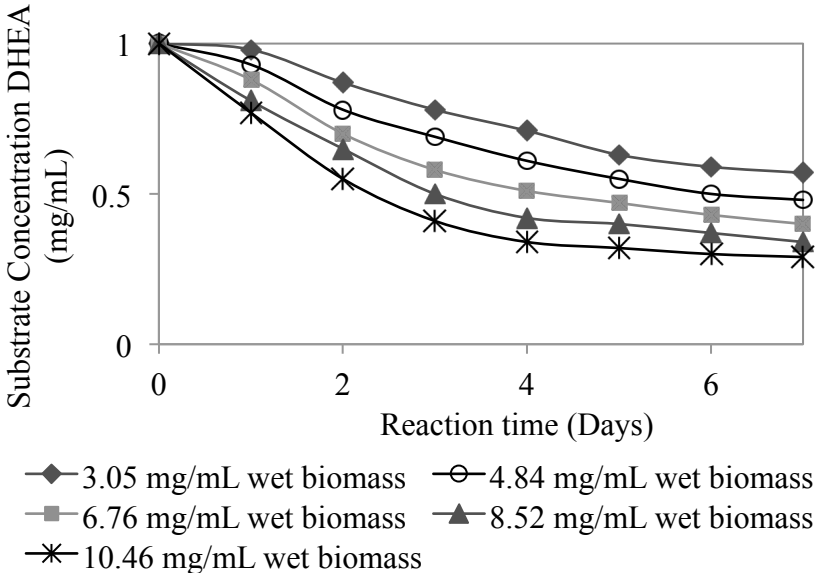


Figure 23: Substrate concentration during the biotransformation with different biomass concentrations.

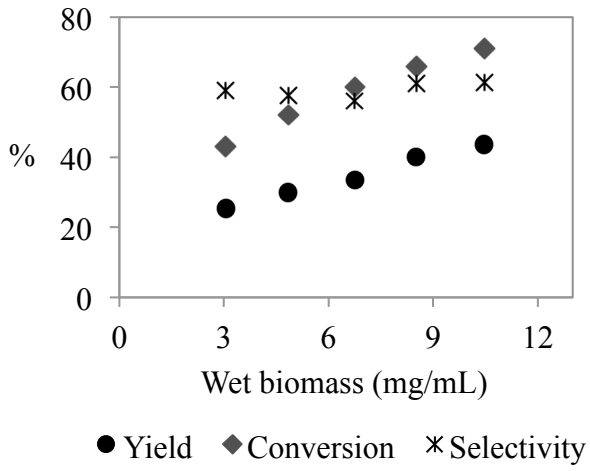


Figure 24: Effect of different biomass concentrations in the conversion of DHEA, selectivity and yield of desired product **II** after 7 days of reaction.

Rate of Reaction Effect with different Biomass and substrate concentrations

Figures 25 and 26 show the results of 7-day biotransformations with different biomass and DHEA concentrations in 100 mL of resting cells. The apparent rate of reaction observed fits a Michaelis-Menten kinetics model (See Figure 25.). Reaction constants were calculated with a non-linear regression (See Appendix A for sample calculations). Low biomass yield (3.04 mg/mL) resulted in a maximum reaction rate V_{max} of 3.02 mM/day and K_M 14.17 mM. Medium biomass yield (6.10 mg/mL) resulted in a maximum reaction rate V_{max} of 2.45 mM/day and K_M 5.76 mM. High biomass yield (10.46 mg/mL) resulted in a maximum reaction rate V_{max} of 2.37 mM/day and K_M 1.95 mM. Values for substrate conversion were determined for each condition.

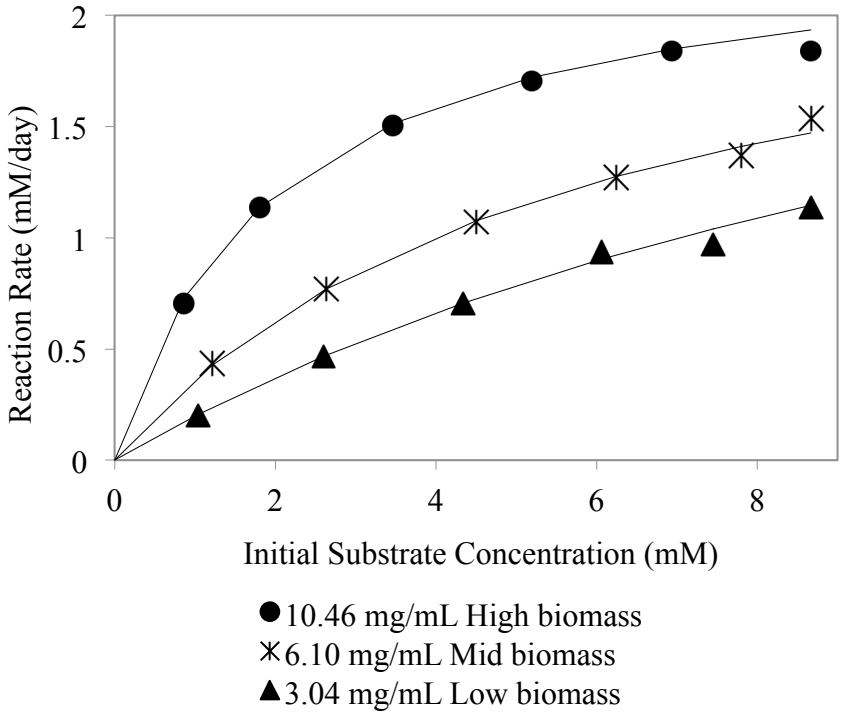


Figure 25: Effect of different biomass concentrations on reaction rate. Faster reaction rates were found with the use of high biomass.

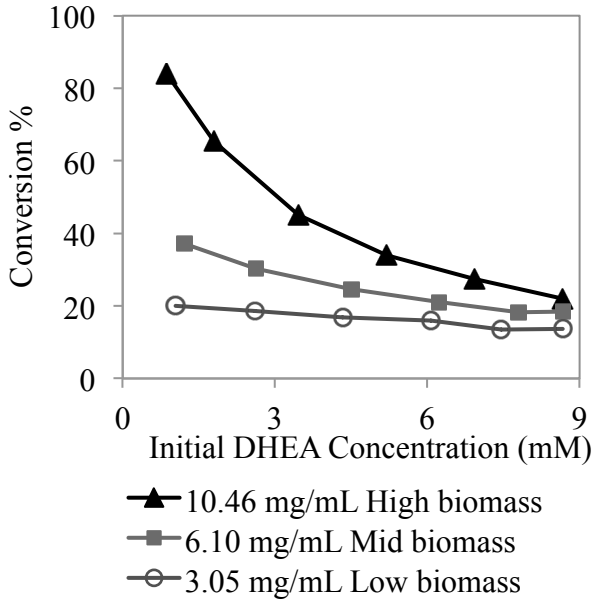


Figure 26: Effect of initial substrate concentrations on the conversion of DHEA with different biomass concentrations.

Discussion

After adapting cells to n-hexadecane, the biomass production and growth rate in a glycerol medium was characterized to control and further advance the potential of n-alkane-adapted *B. bassiana* as a biocatalyst. Measurements of wet biomass are commonly used because of the simplicity of time and experimental procedures. Accurate results were obtained with high amounts of biomass (11.60 mg/mL). A low standard deviation (0.92) was achieved after 13 hours of growth (See Figure 21.). These measurements were taken during the exponential growth between 4 and 13 hours, which fit a linear trend with an R^2 of 0.97. Exponential phase was identified using the growth curve presented in Figure 22. Cells adapted to n-alkanes and inoculated in glycerol achieved a higher maximum optical density and a faster growth rate (0.58 hr^{-1}), than non-adapted cells (0.23 hr^{-1}) (See Figure 22.). This means that the step of adapting cells to n-hexadecane provides a faster and more efficient production of biomass. These differences of biomass yields might be attributed to the possible induction of CYP enzymes caused by the presence of n-alkanes. The use of n-hexadecane as carbon source induces an effective digestion of glycerol medium and thus a faster and higher production of cells.

Strain ATCC 7159 transformed DHEA to androstenediol **I**. Analysis of composition of the product mixtures as a function of reaction time indicates that **I** undergoes an 11α-hydroxylation to synthesize 3,11,17-trihydroxy **II** in all reactions, as shown in Chapter 2 (See Figure 11.). Other by-products were not detected.

The biotransformation of DHEA was carried out under different conditions using five different amounts of biomass. When 1.0 mg/mL of DHEA was subject to reaction, it was found that biomass concentration greatly affects substrate conversion and desired product yield. Experiments showed that the conversion of DHEA increases proportionally to the constant increase of cells in the flask (See Figure 23.). Experiments using a low number of wet biomass like 3.05, 4.84 and 6.76 mg/mL; resulted in 43, 52 and 60% conversion of DHEA respectively. At higher number of wet cells like 8.52 and 10.46 mg/mL, the conversion was 66%, with 71% maximum conversion respectively. After four days of reaction with high biomass, the conversion of DHEA reached a plateau (maximum conversion). This may be caused by various reasons. After 4 days of reaction, the activity of the reductase that synthesized intermediate androstenediol is affected, resulting in a deficient conversion. As well, this may be caused by a competitive inhibition in the reductase. Interestingly, when this plateau is reached in all biotransformations (after 4 days), at the same time the 11,alpha-OH product starts to be detected. Somehow, this product may be related to the inhibition. Furthermore, after 7 days of reaction the increment in desired product yield was directly proportional to the increasing presence of cells (See figure 24.). The yield of **II** was enhanced from 25 to 43.5% with the use of dense biomass. This is an increment of 42.5% by simply extending the time of cells growth from 5 to 12 hours with glycerol medium. The reaction selectivity was constant on 60% through all experiments. Interestingly, the changes in the number of cells does not induce the synthesis of new steroids besides **I** and **II**, neither have a considerable effect on reaction selectivity. This fact can be explained by assuming

that the expression and activity of proteins responsible for the biotransformation of **I** and subsequently **II**; increases proportionally with the amount of biomass produced.

The rate of reaction and the conversion of DHEA during the biotransformation greatly depend on initial substrate concentration. To better understand these parameters, experiments were performed with different initial concentrations of DHEA ranging from 0.25 mg/mL (0.86 mM) up to 2.5 mg/mL (8.66 mM), and biomass yields of 3.04, 6.10 and 10.46 mg/mL. The apparent rate of reaction observed in all experiments fits a Michaelis-Menten kinetics model. The maximum reaction rate (V_{max}) decreased constantly with the use of dense biomass (See Figure 25). The higher V_{Max} achieved was 3.02 mM/day with 3.04 mg/mL of wet cells. As the amount of biomass increased, V_{max} declined proportionally. Interestingly, experiments with diluted amounts of DHEA (0.25 mg/mL) resulted in 84% conversion, which is higher than the 22.6% obtained with high concentrations of DHEA (2.5 mg/mL) (See Figure 26.). This presumably results from the interaction of large number of cells in contrast to a small concentration of DHEA. These results provide the opportunity for modifications in the design of the processing scheme. A fed-batch reactor could be used, in which more substrate is added to the reactor after a substantial amount of initial substrate has been converted. For example, if the initial concentration of substrate is chosen properly at a low range (0.25-1.0 mg/mL), a fast reaction rate and a high conversion could be achieved, and more substrate could be added to the system to increase the conversion. In addition, the value of K_M decreases from 14.17 to 1.95 mM in experiments with 3.04 and 10.46 mg/mL of wet biomass respectively. This means that different reaction kinetics with respect to the amount of

biomass were obtained, and the ratio (V_{max}/K_M), pseudo first order reaction rate, is the basis for the evaluation of the reaction (Sassa 2008 and Radika 1984). A low K_M means the need of a low substrate concentration to achieve maximum reaction rate V_{max} (saturation of the biocatalyst) (Williams 1978). Thus for the biotransformation of DHEA, high V_{max}/K_M values are desired to achieve maximum reaction rates at low DHEA concentrations. This was achieved with the use of dense biomass (See Figure 27.).

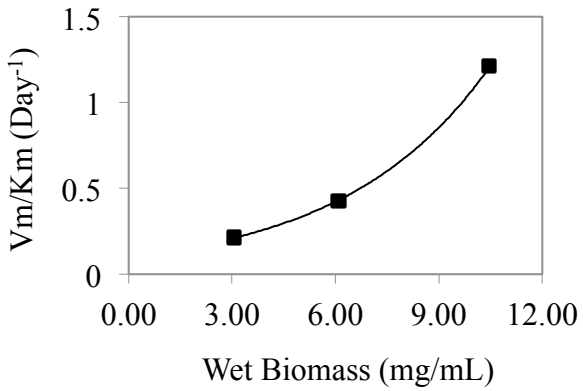


Figure 27: Effect of increasing biomass yields in the parameters of Michaelis-Menten kinetics. A high ratio of V_{max}/K_M is recommendable to achieve a maximum reaction rate at low DHEA concentration.

Experiments combining the amount of biomass and the starting DHEA concentration as independent variables, provided the opportunity to further understand the biocatalytic potential of n-alkane-adapted *B. bassiana*. Experiments with high biomass density and low DHEA concentration resulted in higher substrate conversion (See figure 28.). This can be explained by assuming that the presence of a high number of cells relative to low substrate concentration increases the probability for the reaction to happen. As well, higher initial concentrations of substrate tend to saturate the reaction mechanism, as it was demonstrated in Chapter 3.

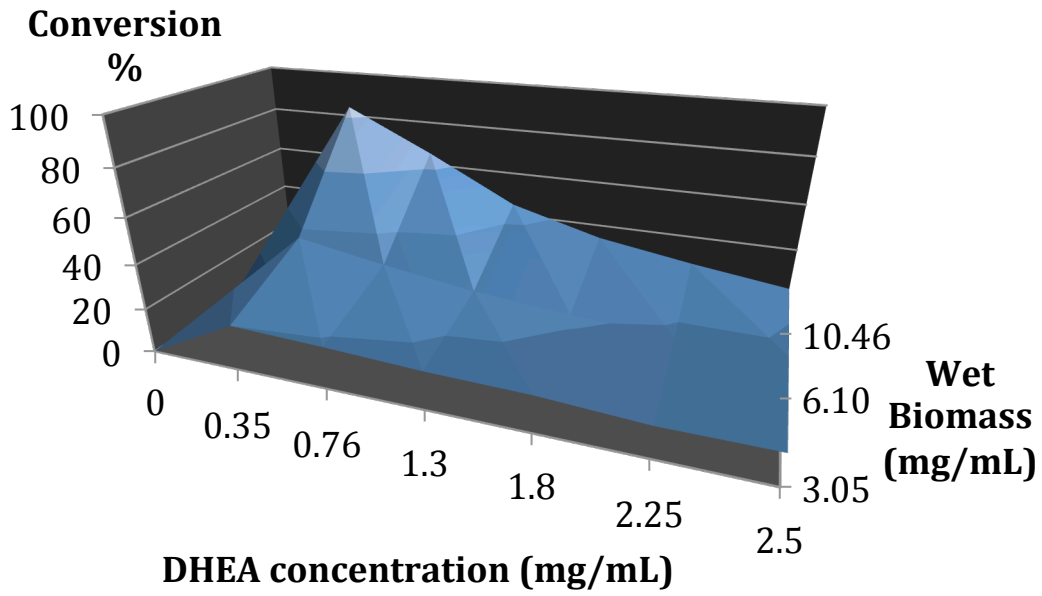


Figure 28: Design of experiments with different number of cells and substrate concentration. These two variable factorial experiments showed that optimal production of steroids was achieved at low starting concentration of DHEA and a dense biomass culture.

Conclusions

It was shown that cells adapted to n-hexadecane before the inoculation of glycerol provide a faster and efficient production of biomass. Further, two types of oxidative products were observed during the biotransformation of DHEA and its conversion increased proportionally to the amount of biomass used. The maximum conversion reached (71%) was achieved in experiments with high biomass, and the increment of desired product yield is directly proportional to the amount of biomass and its growing time. Interestingly, the changes in biomass concentration do not have a considerable effect on reaction selectivity. A high V_{Max}/K_M value was achieved with high biomass concentrations and the rate of reaction increased proportionally to higher concentrations

of cells. Optimal production of steroids was achieved at low initial concentration of DHEA and dense biomass. The synthesis of 11α-DHEA was enhanced when cells were previously adapted to n-hexadecane, and more importantly, these results suggest that this fungus presents a promising future as biocatalyst to be used for enhancement in the production of steroids.

CHAPTER 5
ANALYSIS OF PUBLISHED N-ALKANE INDUCED CYTOCHROME P-450 GENES IN N-ALKANE ADAPTED CELLS OF *BEAUVERIA BASSIANA* ATCC 7159

Introduction

In previous Chapters, we explored the synthesis of steroids with *B. bassiana* as a biocatalyst, but still, poor information is known about the enzymatic activity of this fungus. In Chapters 2, 3 and 4, we established robust experimental arrangements for the biotransformation but which specific enzymes are responsible for the reduction of DHEA and eventually the 11,alpha-oxidation of androstenediol is still unknown. In this Chapter, we present preliminary results and the future work in the characterization of the biomolecular machinery of *B. bassiana.* The protocols for DNA extraction, gene sequencing and eventually the identification of expressed genes were developed. These tools will be useful to identify the genes expressed when *B. bassiana* strain ATCC 7159 is grown in the presence of n-alkanes; and to label the proteins that are actively involved in the reaction. It is important to characterize these oxidative enzymes to facilitate the production of 11,alpha hydroxyl steroids. By sequencing these genes, we could use recombinant DNA for specific expression of these enzymes to avoid non-desired products from other types of enzyme-catalyzed reactions.

Experimental

Preparation of fungal mycelia

Starting cell stock was grown on an n-alkane medium (NM). A 10% v/v of the NM culture was transferred into 200 mL of Glycerol medium (GM) (see Chapter 2 - Media preparation). Aliquots (1mL) of previously grown cells were used to inoculate glycerol-agar plates: glycerol (0.1 L), Corn Steep Liquor (20 g/L), deionized water (1 L), pH 7, and agar (15 g/L). Plates were incubated for 4 days at 26°C.

Extraction of fungal DNA

Mycelium grown on the surface of agar plates was removed by scraping the surface with a sterile scalpel. A single colony was removed from the agar, freed from traces of agar, suspended in 1mL of distilled water in an Eppendorf tube and fragmented by pipetting. After washing twice with distilled water in an Eppendorf tube, the mycelia were freeze-dried (lyophilized) and stored at −20 °C until used. Freeze-dried mycelium was ground to a fine powder with liquid nitrogen using a pre-cooled pestle. DNA was extracted from the pestle following the protocol of a SIGMA Plant/Fungi DNA Isolation Kit. The extracted DNA was washed three times with 70% ice-cold ethanol, dried and dissolved in 50 mL Tris-EDTA (TE) buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA pH 7.8) and stored at −20°C.

PCR Protocol

Primer design: Primers were design at Integrated DNA Technologies (IDT) following characterized P-450 genes in Pedrini et.al (Pedrini 2010). Eight primers

(forward and reverse) were designed for Rapid amplification of cDNA ends (RACE) and Reverse transcription polymerase chain reaction (RT-PCR) assays. Primers were designed for CYP genes 2, 3, 6 and 7. The gene expression of these CYP enzymes was highly elevated in the presence of n-hexadecane (Pedrini 2010). See gene names and primer sequences in Appendix Q.

10X Primer mix preparation (2 µM of each primer): RACE primers, 90 nmol forward and 90 nmol reverse primers, were diluted with 900 µL H_2O respectively. 1.2 µL of each stock solution was diluted with 47 µL of H_2O to prepare a 10X primer mix of 2 µM of each primer.

All amplifications were performed in a thermocycler and QIAGEN Multiplex PCR Kit. The PCR reaction mix (final volume 50 µL): 15 µL H_2O free RNA, 5 µL DNA template, 5 µL primer mix (0.2 µM of each primer), 25 µL PCR Master Mix. This sample was incubated in the thermocycler with PCR cycling program (see Appendix R).

Gel Electrophoresis

Amplification products were separated by electrophoresis in 1.5% agarose gels in 1 × TAE buffer for 60 minutes at 125 V, stained with SafeView nucleic acid stain visualized under UV light. The Gene-ruler™ DNA ladder mix (10 µL) was used for electrophoresis.

Preparation of Agarose Gel: Agarose was added to a suitable container (250 mL Erlenmeyer flask) with the appropriate amount of 1x electrophoresis buffer to suspend

the Agarose powder in the buffer (e.g. For a 1.5% Agarose gel, 1.5 g of Agarose was added to 100 mL of 1x electrophoresis buffer). The solution was swirled and heated until all of the small translucent Agarose particles were dissolved. The molten Agarose was cooled to 60°C before pouring an Agarose gel slab.

Casting Agarose Gel: Gels were cast with the aid of the gel caster (7 cm x 7 cm casting tray) and standard laboratory tape. Laboratory tape was added on both edges of caster to seal the open tray ends. Combs were put into the appropriate slot(s) of the tray. When the agarose solution was cooled to 50-60°C, the molten Agarose was poured on the tray. The gel was allowed to cure for 20 mins and combs removed after solidification of gel.

Electrophoresis Setup and Run: Electrophoresis buffer 1X Tris-Acetate-EDTA (TAE) buffer was prepared by adding 20 mL of 50X TAE buffer from Thermoscientific to 980 mL of deionized water. 1 µL of loading dye to 5 µL of sample was loaded into the well using standard pipets. The loading dye was "Gel Loading Dye, Blue (6X)" from Thermoscientific.

Ethidium Bromide Staining Procedure after Electrophoresis: When adequate migration occurred, DNA fragments were visualized by staining with ethidium bromide for 30 minutes. This fluorescent dye intercalates between bases of DNA and RNA. The gel was placed into the appropriate volume of 0.5 µg/ml ethidium bromide (EtBr) stain. To visualize DNA or RNA, the gel is washed for 30 minutes with water and placed on an

ultraviolet transilluminator.

DNA Extraction from agarose gel, sample preparation and analysis: A scalpel blade was used to cut around the band of interest. The gel DNA was extracted by following the protocol of QIAquick Gel extraction kit. On a 0.5 mL centrifuge tube, 10 µL of the amplified gene extracted from the gel was added, with 3 µL of 5 pmol/µL of ONE primer stock solution. To prepare this stock solution, 2.5 µL from 100 pmol/µL (0.100 nM/µL = 102.8 µM) primer was diluted on 47.5 µL of H_2O. The prepared samples were sent for sequencing at the Iowa Institute of Human genetics at The University of Iowa.

Preliminary Results

To verify the presence of CYP genes in *B. bassiana*, the DNA was extracted and the prepared primers were amplified. In the QRT-PCR method, the primer for gene P-450 #2 (CYP655C1) was successfully amplified. The results of gel electrophoresis showed two amplified bands with this primer (See Figure 29). Bands were noticed at 250 and 550 base pairs (bp). Other genes were not observed. Aliquots (40 µL) of amplified gene for each sample was prepared and sent to sequencing analysis.

For the 250 bp sample, the sequence that produced significant alignment was *Burkholderia cepacia* GG4 chromosome 1. The identity match was 89% and has been reported at Hong et. al. (Kar-Wai 2012). For the 550 bp sample, the sequence that produced significant alignment was *Propionibacterium acnes.* The identity match was 89% and has been reported at "Complete genome sequence of *Propionibacterium acnes*

type IB strain 6609" (Hunyadkürti 2011) (See Appendix S for sequence.).

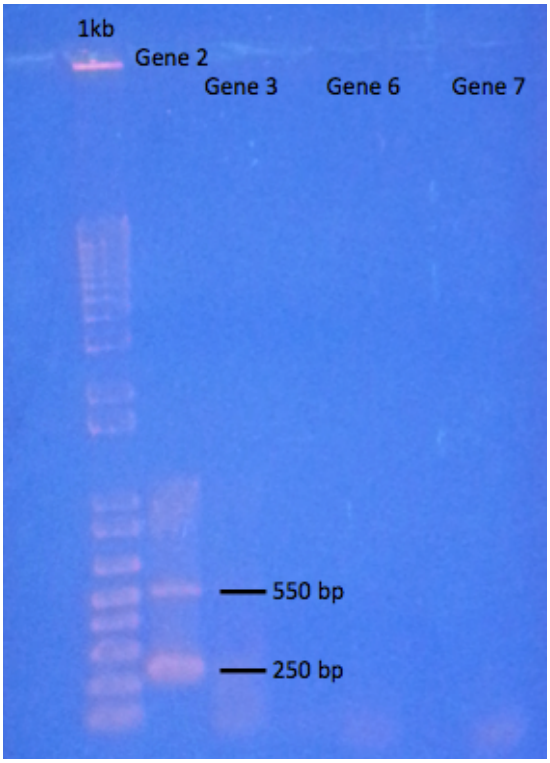


Figure 29: The visualization of various amplified genes showed that only P-450 gene 2 is present in *B. bassiana* strain ATCC 7159 at 250 and 550 bp.

With these results we confirm that from the eight (8) genes that were reported to be overexpressed with n-alkanes, only gene CYP655C1 was identified in n-alkane adapted *B. bassiana* ATCC 7159.

Summary and Future Studies

The genetic expression of CYPs in two major conditions needs to be compared: cells adapted to n-alkanes and non-adapted cells. It would also be informative to evaluate whether CYPs are overexpressed after a biotransformation (cells being exposed to the

substrate DHEA). An RNA extraction and analysis is needed to confirm if this gene is expressed in the system and if it is responsible for the hydroxylation reaction. Future genetic analysis is needed just to confirm or identify which *B. bassiana* CYPs are being overexpressed in the presence of n-alkanes and how to connect these genes to the enzymes responsible for steroid conversion. In Chapter 6, there is a description of the future work and recommendations in enhancing the overall biotransformation.

CHAPTER 6
FUTURE WORK IN BIOCATALYSIS WITH N-ALKANE ADAPTED *BEAUVERIA BASSIANA*

Biotransformation with similar substrates

The hydroxylation of an unactivated carbon in substrate DHEA was performed using *B. bassiana* ATCC 7159. Therefore, same reaction can be studied with other substrates to gain a better understanding of the breadth of hydroxylation capacity within this strain of *B. bassiana.* The activation of an unactivated carbon with *related substrates* (see Chapter 1), could provide an insight in the biocatalyst performance when it comes to different chemical structures. Moreover, the optimal parameters established in Chapter 3 could be applied for the production of other metabolites. In addition, other types of reactions such as sulfoxidations, reductions, and epoxidations can be explored with this biocatalyst and these experimental arrangements.

Bioreactor Studies

This thesis presented optimizations of batch processing. Further work can be realized upon scaling up the process to a fermenter to improve the reaction. High cell densities can be used in the bioreactor to further improve the productivities. Control of mixing oxygen mass transfer, pH, temperature, cells number, agitation and medium composition, within a bioreactor may enhance productivities. As well, maintaining optimum conditions and required concentration of nutrients is necessary to maintain the viability/activity of the biocatalyst. Further studies can be done with the opportunities to

run the biotransformation in fed-batch or continuous mode. A spike of substrate (more addition of substrate besides the initial concentration) after a few days of reaction can be used to overcome limiting rates and current maximum conversion.

Cells Immobilization and Recycle

Recycle of the biocatalyst is often necessary to improve the efficiency and economics of bioprocesses. Immobilization may improve the stability of the biocatalyst by maximizing direct contact of cells with nutrients and substrate (Leon 1998). Cells immobilization can maximize direct contact with the organic (n-alkane) phase, thus increasing the expression of CYPs and avoiding a biphasic cell growth. However, immobilization introduces mass transfer limitations associated with transport of substrate and product across the immobilization matrix.

Process modeling and simulations

Computational approaches can be used to predict optimal experimental parameters and potential results of a biotransformation. Programs like Aspen can be used to simulate and optimize this batch operation, and to design future fed-batch work. As well, programs like MatLab and QI Macros can be used for statistical analysis, considering multiple factors as independent variables at the same time. In this work, we considered the impartial effect of multiple variables like temperature, pH, agitation and culture medium. By using a statistical approach and a Design of Experiments (DOE)

dependent of multiple variables at the same time, a deeper understanding could be achieved on identifying which variable has the bigger impact on the biotransformation.

Enzyme studies

All our work was done with *B. bassiana* as a whole-cell biocatalyst and none was done with just enzymes. The yield of desired metabolites in the actual process could be enhanced with the use of specific oxidative enzymes and co-factors. Therefore, room for improvement exists in the purification, recycle and immobilization of specific oxidative enzymes, for the biotransformation. To enhance and overcome the overall conversion of DHEA to desired 11,alpha-OH steroid; a powerful overexpression, separation and concentration of hydroxylases is needed to catalyze faster reactions and greater loads of intermediate androstenediol (Martinez 2013). In addition, it would be useful to feed reduction cofactors to see if the limitation on the DHEA step can be overcome.

Cells n-alkanes adaptation

Cells were adapted and successfully grown in a n-alkane medium (see Chapter 2). After 59th-cycle of cells grown in n-hexadecane, still is not clear if the new environment of organic solvents can modify the genetics of strain ATCC 7159. A phenotype analysis is recommended to understand the genetics changes; performance of the biocatalyst, and to corroborate if in fact strain ATCC 7159 has not changed significantly.

CHAPTER 7
CONCLUSIONS

The activation of unactivated carbon 11 in DHEA was enhanced when the substrate was subjected to biotransformation with cells adapted to n-hexadecane. Two types of oxidative products were observed in all biotransformations, however the appearance of the products suggests that the reduction of the C-17 ketone of DHEA precedes the 11α-hydroxylation. Desired 11,alpha-hydroxy steroids were synthesized more efficiently in a resting cell arrangement with a GM/Buffer solution ratio of 4, with agitation at 250 RPM, with moderate DHEA concentration (0.83-1.0 mg/mL), and with a mild environment (26°C, pH 7). The limiting step (slowest) in the reaction was determined to be the transformation of intermediate androstenediol (**I**) to desired 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

Results showed that substrate conversion increased proportionally to the number of wet cells used. The maximum conversion reached (71%) was achieved in experiments with high biomass, and the increment of desired product yield is directly proportional to the amount of biomass and its growing time. Interestingly, the changes in biomass concentration do not have a considerable effect on reaction selectivity. A high V_{Max}/K_M value was achieved with high biomass concentrations and the rate of reaction increased proportionally to higher concentrations of wet cells. Optimal production of steroids was achieved at low initial concentration of DHEA and a dense biomass culture. In addition, from the genes that are highly expressed with n-alkanes, only P-450 gene CYP655C1 is present in strain *B. bassiana* ATCC 7159.

These results show the ability of *B. bassiana* to adapt and metabolize a substrate in different biotransformation settings. More importantly, these results suggest that this fungus presents a promising future as biocatalyst to be used for enhancement in the production of drug metabolites. We successfully developed a more efficient process to activate an unfunctionalized carbon in steroids. Moreover, the use of a biodegradable biocatalyst converts this approach into an attractive eco-friendly technique. Further, a well-characterized biocatalyst supports industries engaged in the production of more expensive drugs.

The process modifications presented herein will help in addressing problems associated with production of valuable products using biotransformations. This research served as an experience and motivation for four undergraduate students, and resulted in two manuscripts, twelve presentations at national conferences and six presentations at local conferences (see Appendix T).

APPENDIX A. SAMPLE CALCULATIONS

After 7 days of a flask reaction of 200 mg of DHEA in 200 mL of medium, the recovery of metabolites resulted in a dry weight of 195.8 mg. For the preparation of Sample A, the 195.8 mg previously recovered was diluted in 15 mL of methanol–water solution (60:40, v/v). This solution was further diluted to 75 mL for HPLC analysis. The HPLC results of the extracted crude resulted in three distinctive peaks. The area under the curve (AUC) for each peak was previously characterized and identified as P for unreacted substrate (AUC 286060), P1 as intermediate androstenediol (**I**) (AUC 471990), and P2 as desired product 11,alpha,OH-androstenediol (**II**) (AUC 221680).

Table A1: Molecular weight of steroids

Steroid	Molecular Weight (g/mol)
Molecular mass of androstenediol	290.44
Molecular mass of 11OH-androstenediol	306.44
Molecular mass of DHEA	288.424

Part 1: Calculations of the concentrations from HPLC analysis

With the linear regression of the calibration curve of each metabolite (See Appendix B), the concentration (mM) in the sample was calculated. For example, the calibration curve for Ps is

$$y = 101570x + 9852.1$$

The calibration curve for P1 is

$$y = 119899x - 13899$$

and the calibration curve for P2 is

$$y = 127562x + 13899$$

Solving the calibration equation for Ps with an AUC of 286060, the absolute DHEA amount is:

$$x = \frac{286060 - 9852.1}{101570} = 2.71\ mM$$

For P1 is 4.05 mM and for P2 is 2.08 mM. Since the sample was diluted from 15 mL to 75 mL for HPLC analysis, a dilution factor (D) was used. This is calculated as follows:

$$D = \frac{V_2}{V_1} = \frac{75\ mL}{15\ mL} = 5$$

Therefore, the final amount of Ps in sample A was:

$$Ammount\ of\ Ps\ in\ sample\ A = 2.71\ mM\ x\ 5 = 13.55\ mM$$

The amount of P1 in sample A was 20.25 mM and 10.4 mM for P2.

Part 2: Calculation of moles recovered

With these concentrations and the volume of sample A (0.015 L), the moles of Ps recovered after the reaction was calculated as

$$moles\ of\ Ps = 13.55\ mM\ x\ 0.015\ L = 0.203\ mmol$$

The moles of P1 recovered after reaction was 0.306 mmol and 0.156 mmol for P2.

Part 3: Calculation of grams recovered

The grams of Ps recovered after the reaction was calculated as

$$grams\ of\ Ps = mmol\ of\ DHEA\ x\ molecular\ weight\ of\ DHEA$$

$$grams\ of\ Ps = 0.203\ mmol\ x\ 288.42\ \frac{mg}{mmol} = 58.65\ mg$$

The amount of P1 recovered after reaction was 89.65 mg and 47.94 mg for P2. It means that from 200 mg of starting DHEA, 58.65 mg of DHEA did not react, and 89.65 mg of **I** and 47.94 mg of **II** were synthesized. The sum of calculated metabolites (P_{total}) should approximate the total recovered (195.4 mg). As a double check:

$$P_{total} = Ps + P1 + P2 = 58.65\,mg + 89.65\,mg + 47.94\,mg = 196.24\,mg$$

Part 4: Calculation of conversion, selectivity and yield

The conversion of DHEA and the Selectivity of desired product **II** over byproduct **I** (intermediate)**,** were calculated as

$$Conversion_{DHEA} = \frac{Moles_{\ Reacted}}{Moles_{Initials}} = \frac{0.693\,mmol - 0.203\,mmol}{0.693\,mmol}\ x\ 100 = 70.7\%$$

$$Selectivity_{11a-OH\ Product} = \frac{Moles_{\ 11a-OH\ Product}}{Moles_{Byproduct\ II}} = \frac{0.156\,mmol}{0.306\,mmol}\ x\ 100 = 50.9\%$$

For a batch reactor (flask in our case), the combination of conversion and selectivity can be used to calculate the yield of desired product **II.**

$$Yield_{11a-OH\ Product} = Conversion_{DHEA}\ x\ Selectivity_{11a-OH\ Product}$$

$$Yield_{11a-OH\ Product} = 0.707\ x\ 0.509\ x\ 100 = 35.9\%$$

Part 5: Calculation of rate of reaction

To calculate the concentrations needed for the Michaelis-Menten curve fitting, the kinetics of the reaction was measured as a function of the initial concentration of DHEA available to the cells. Samples were taken each day of reaction. HPLC analysis was performed on these samples and the concentration of formed product **I** and **II** were

measured. For 200 mg of DHEA in 200 mL of medium, the concentration of initial substrate was calculated as

$$Concentration\ of\ substrate = \frac{1\ mg/mL\ of\ DHEA}{288.42\ \frac{mg}{mmol}}\ x\ 1000\ mL/L = 3.46\ mM$$

The moles of P1 and P2 recovered each day were calculated as shown in Part 2, and the rate of reaction was calculated as

$$v = \frac{d[P_{Total}]}{dt} = \frac{d[P_1]}{dt} + \frac{d[P_2]}{dt} = \frac{d[{}^{mmol_1}/_{0.2L}]}{Day\ 2 - Day\ 1} + \frac{d[{}^{mmol_2}/_{0.2L}]}{Day\ 2 - Day\ 1} = {}^{mM}/_{Day}$$

Part 6: Calculation of intermediate reaction constant

Equation 10 was used to calculate k_1 (reaction constant of the transformation of DHEA to intermediate androstenediol), with previously determined kinetic values. V_{Max} (4.45 mM/day) and K_M (4.86 mM) were calculated with a non-linear regression of the overall reaction (See Chapter 3). V_2 (2.08 mM/day) was calculated in Part 5. For a biotransformation of 25 mg of DHEA in 25 mL of buffer solution (3.46 mM), resulting in 11.2 mg of unreacted DHEA (1.55 mM), 8.1 mg of intermediate **I** (1.11 mM), and 5.7 mg of desired product **II** (0.74 mM). Using equation 10 and known values:

$$v_{overall} = \frac{V_{max}\ [S]}{k_M + [S]} = k_1\ [S] - k_2\ [P_1] + k_2\ [P_1]$$

$$\frac{(4.45\ mMday)[3.46mM]}{4.86\ mM + [3.46mM]} = k_1\ [3.46mM] - 4.3mM[1.11mM] + 4.3mM[1.11mM]$$

Solving for k_1:

$$k_1 = 0.53\ day^{-1}$$

Part 7: Calculation of growth rate

To calculate the growth rate (μ) of *B. bassiana*, the exponential phase of the growth curve was used (See figure 22.). For example, in cells adapted to n-alkanes, the exponential phase was between 4 and 13 hours. The optical density detected at 4 and 13 hours was 2.52 and 7.74 respectively. Samples were diluted 1/10 with water after an OD of 1.5. The OD observed was multiplied by 10, to calculate the real OD of the sample. The slope in this phase was calculated as:

$$\mu = \frac{7.743 - 2.52}{13\ hrs - 4\ hrs} = 0.58\ hr^{-1}$$

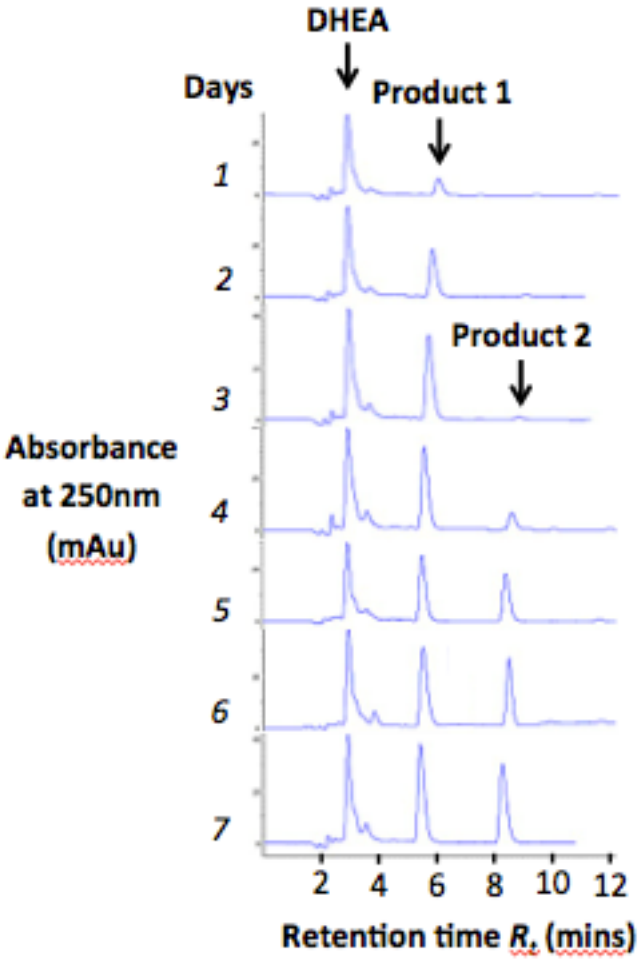


Figure B1: HPLC results for 7 days biotransformation with n-alkane induced cells shows retention time of substrate DHEA (2.96 min), and products **I** (5.23 min) and **II** (8.31 min).

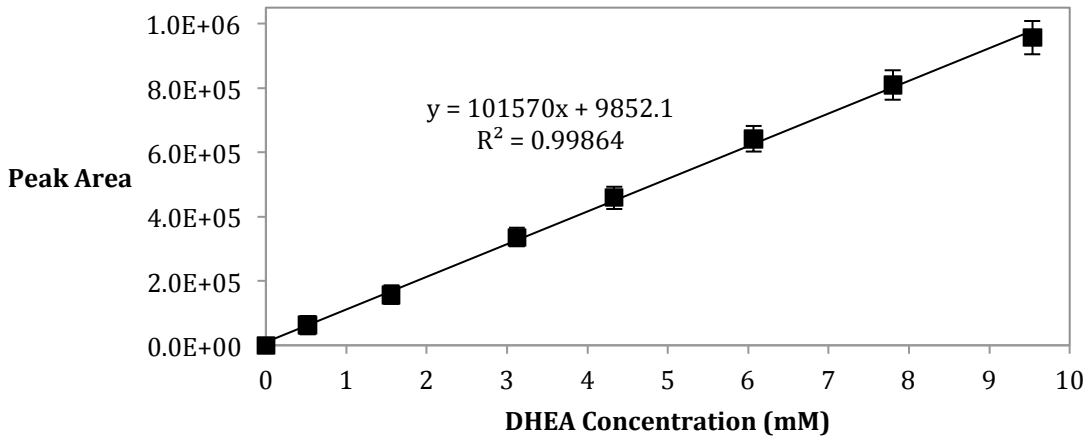


Figure B2: The HPLC calibration curve for substrate DHEA. Results are means ± Std Dev., n = 3.

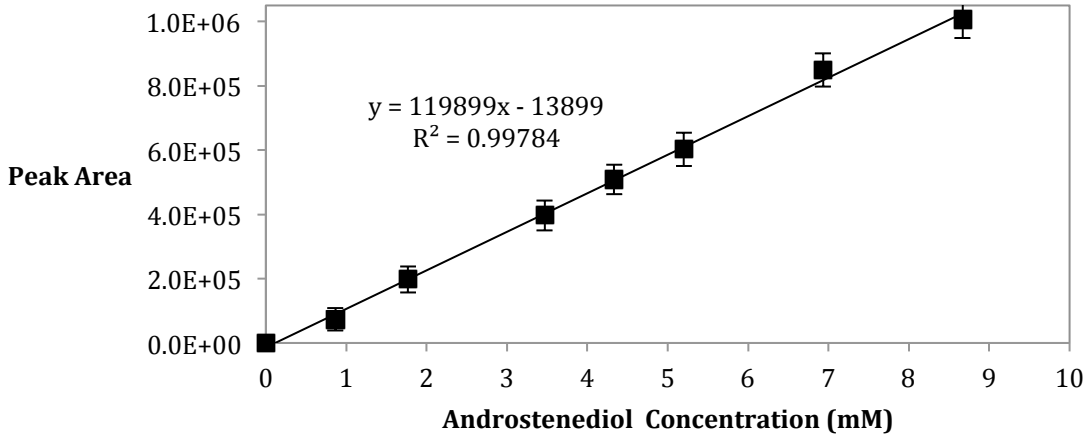


Figure B3: The HPLC calibration curve for androstenediol (**I**). Results are means ± Std Dev., n = 3.

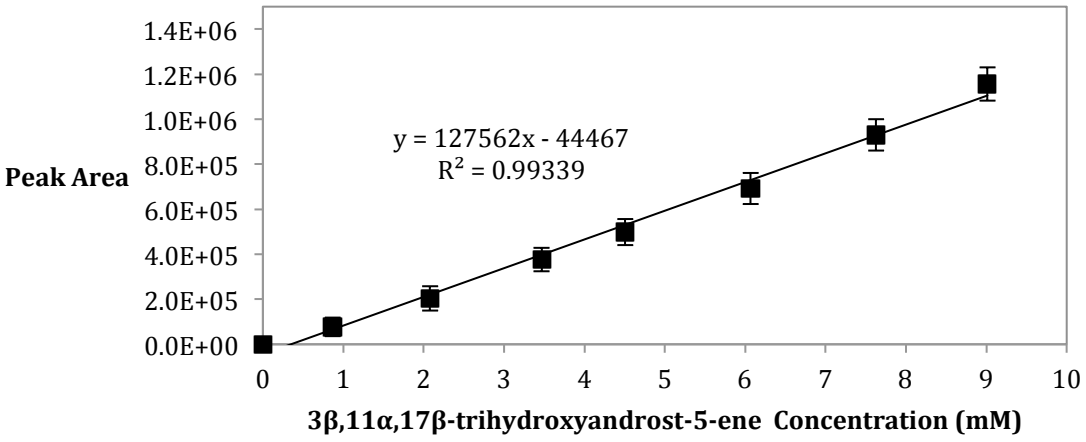


Figure B4: The HPLC calibration curve 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

Results are means ± Std Dev., n = 3.

APPENDIX C. ^{1}H NMR SPECTRA

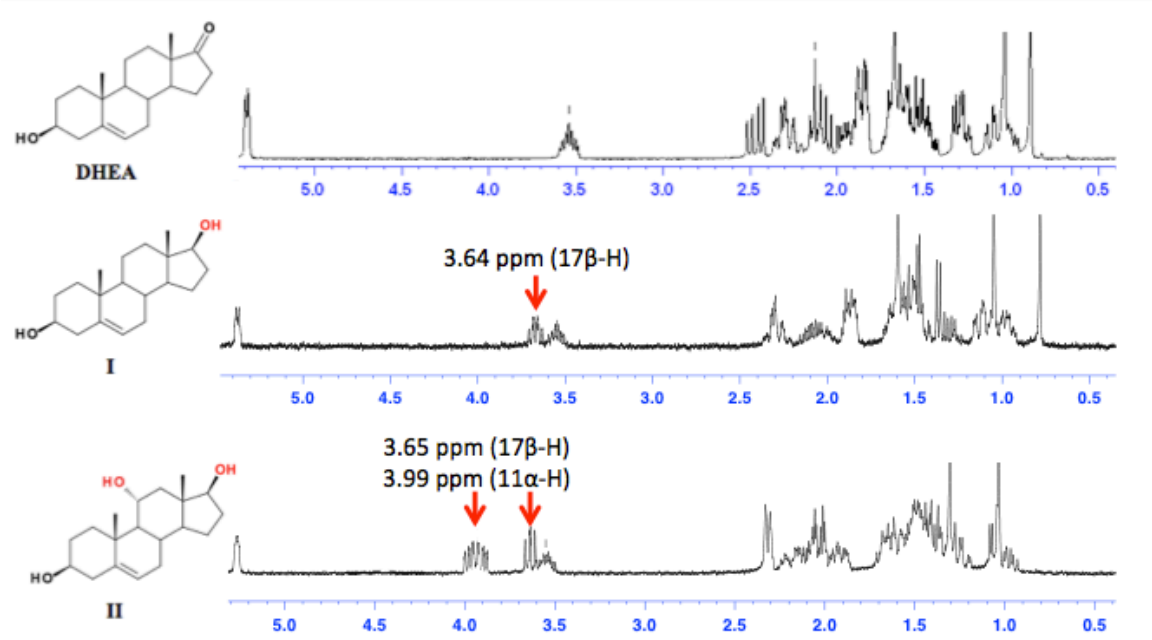


Figure C1: Oxidation characteristic peaks in the Proton NMR spectra of substrate DHEA and products androstenediol (**I**) and 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

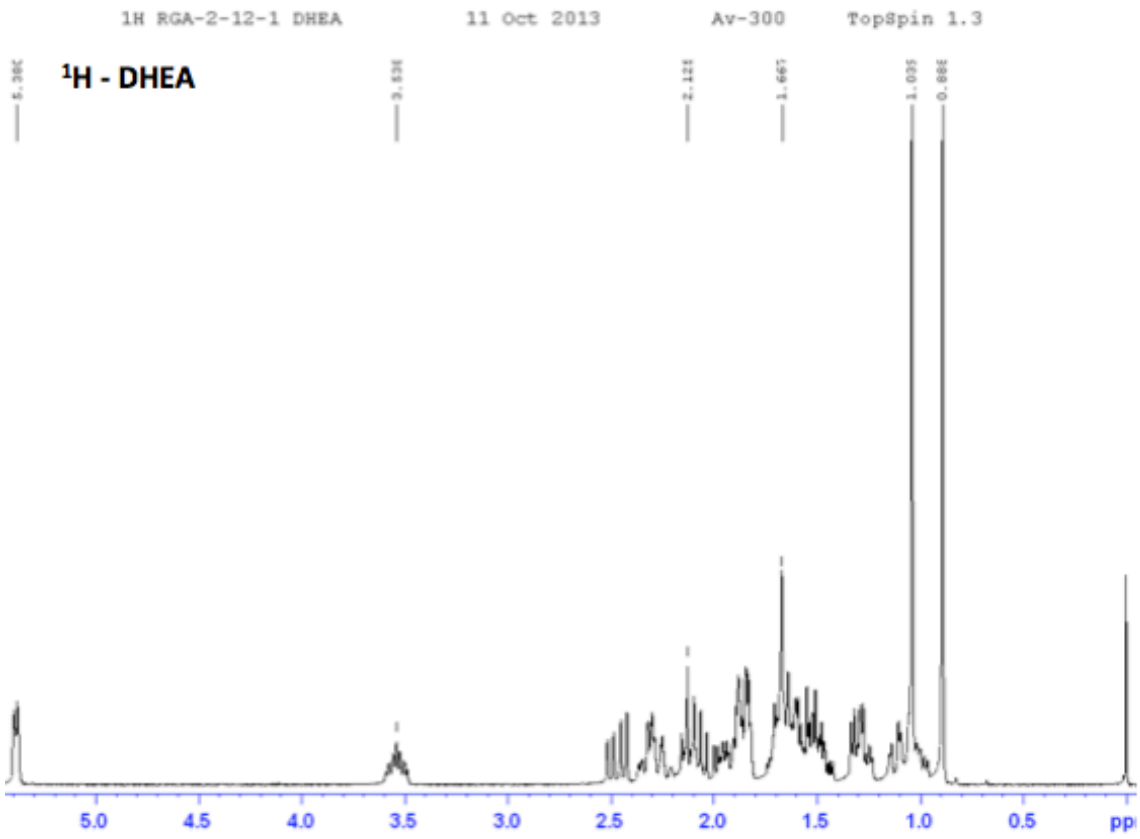


Figure C2: Proton NMR spectrum of substrate DHEA.

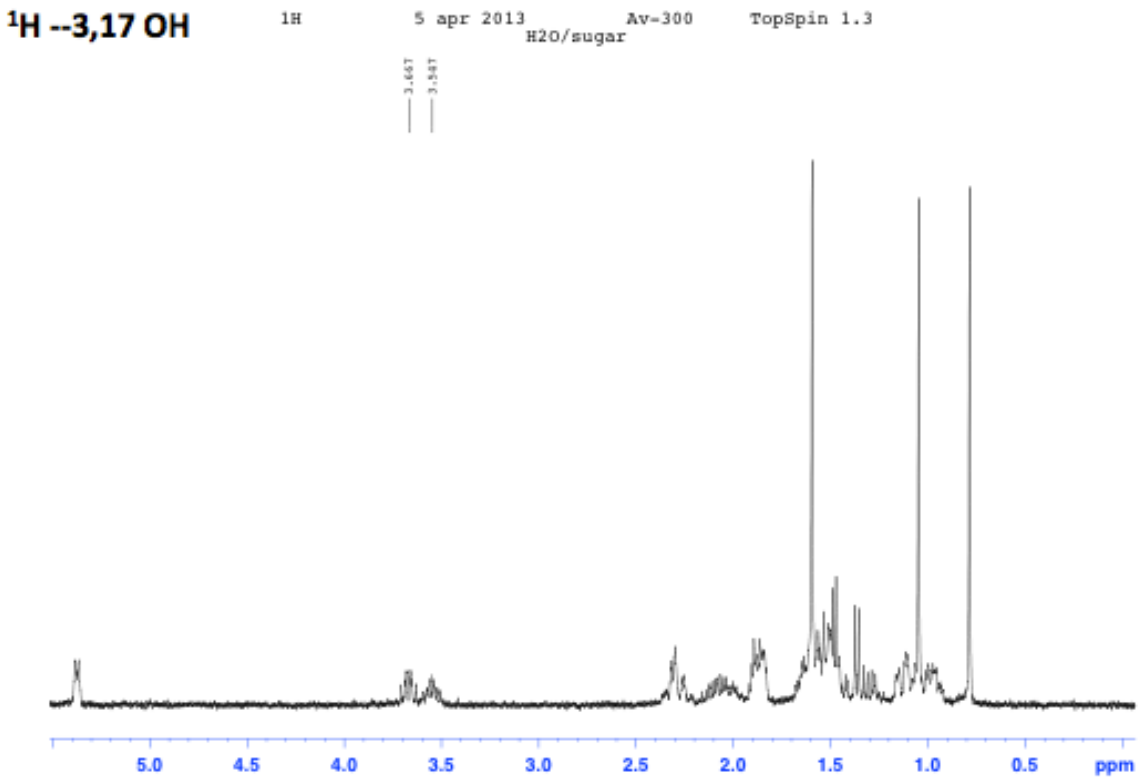


Figure C3: Proton NMR spectrum of androstenediol (**I**).

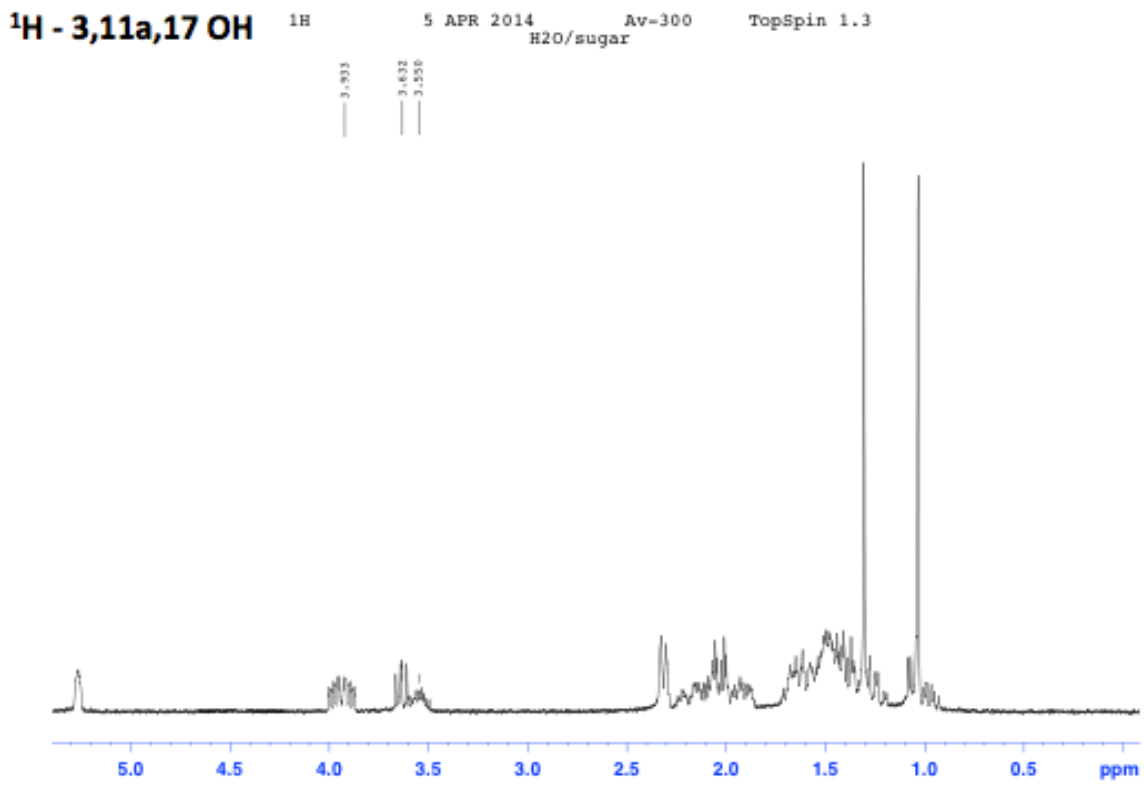


Figure C4: Proton NMR spectrum of 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

APPENDIX D. ^{13}C NMR SPECTRA

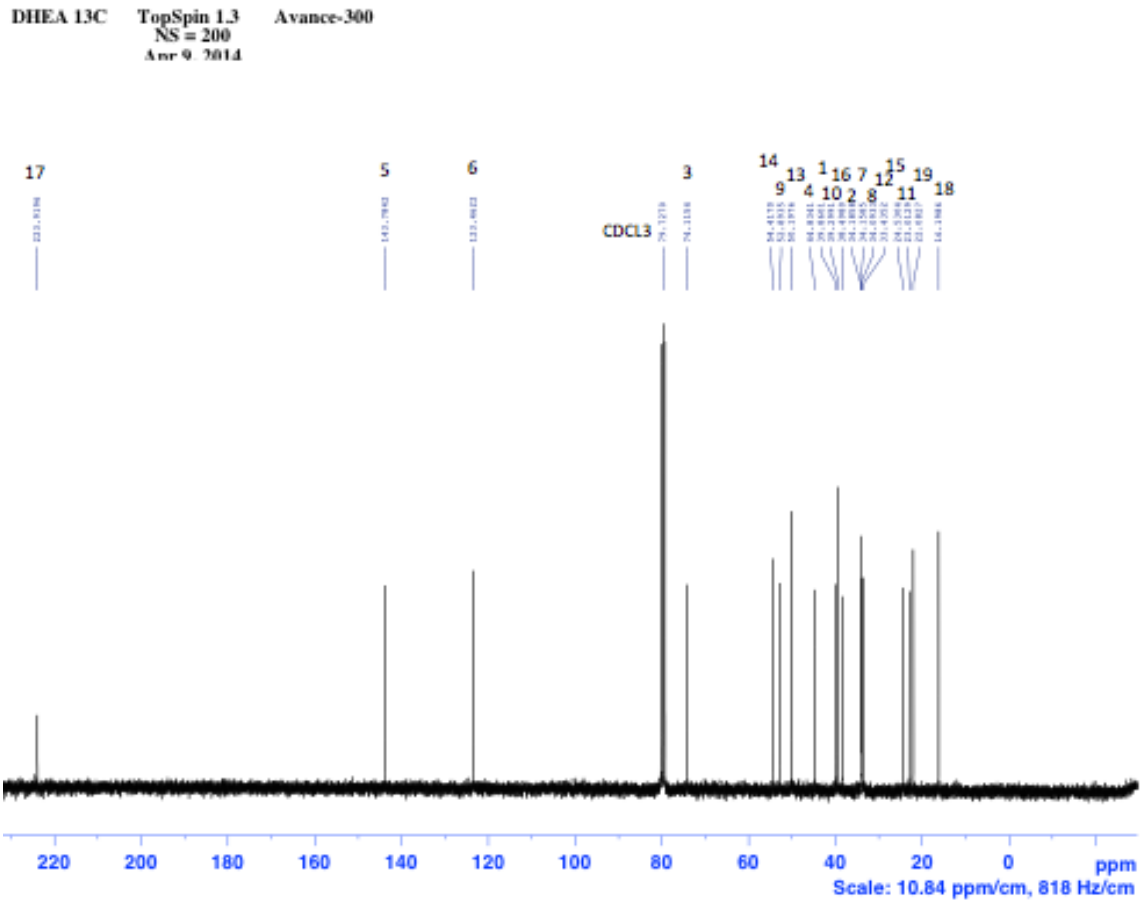


Figure D1: Carbon NMR spectrum of substrate DHEA.

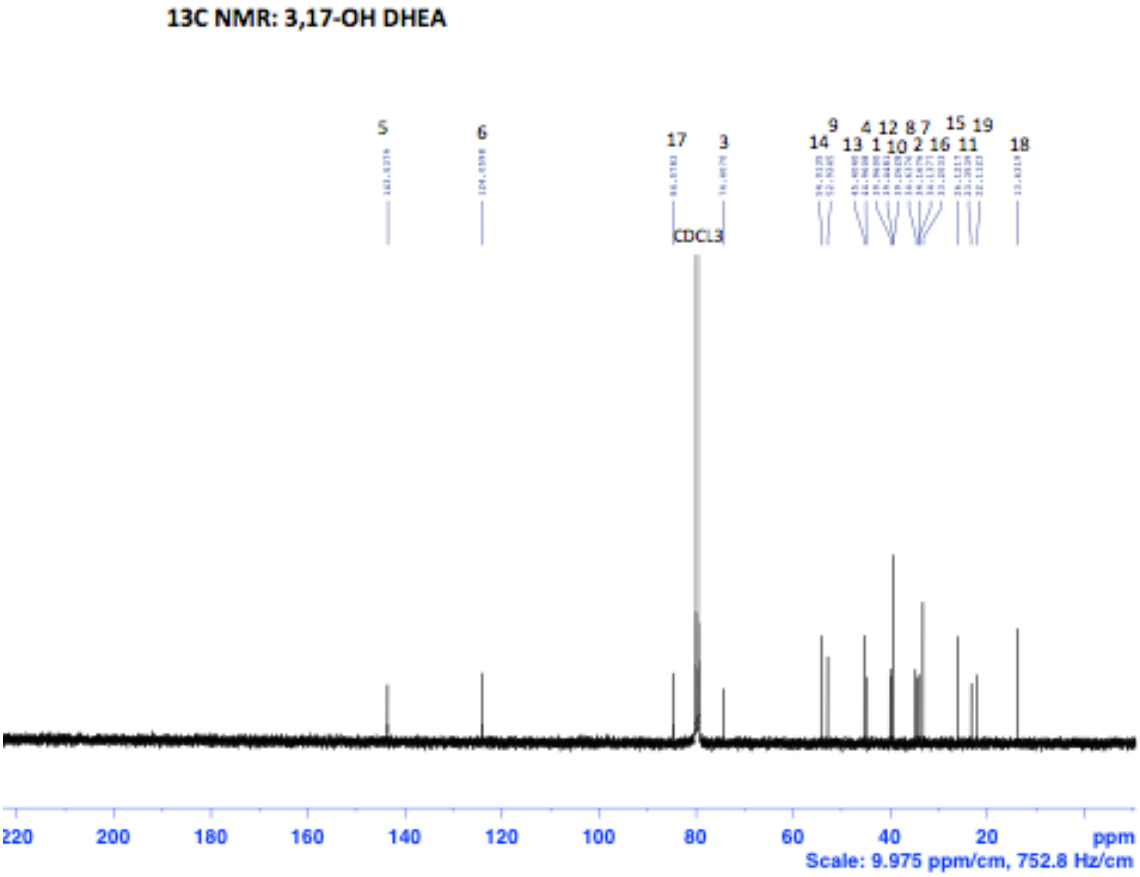


Figure D2: Carbon NMR spectrum of androstenediol (**I**).

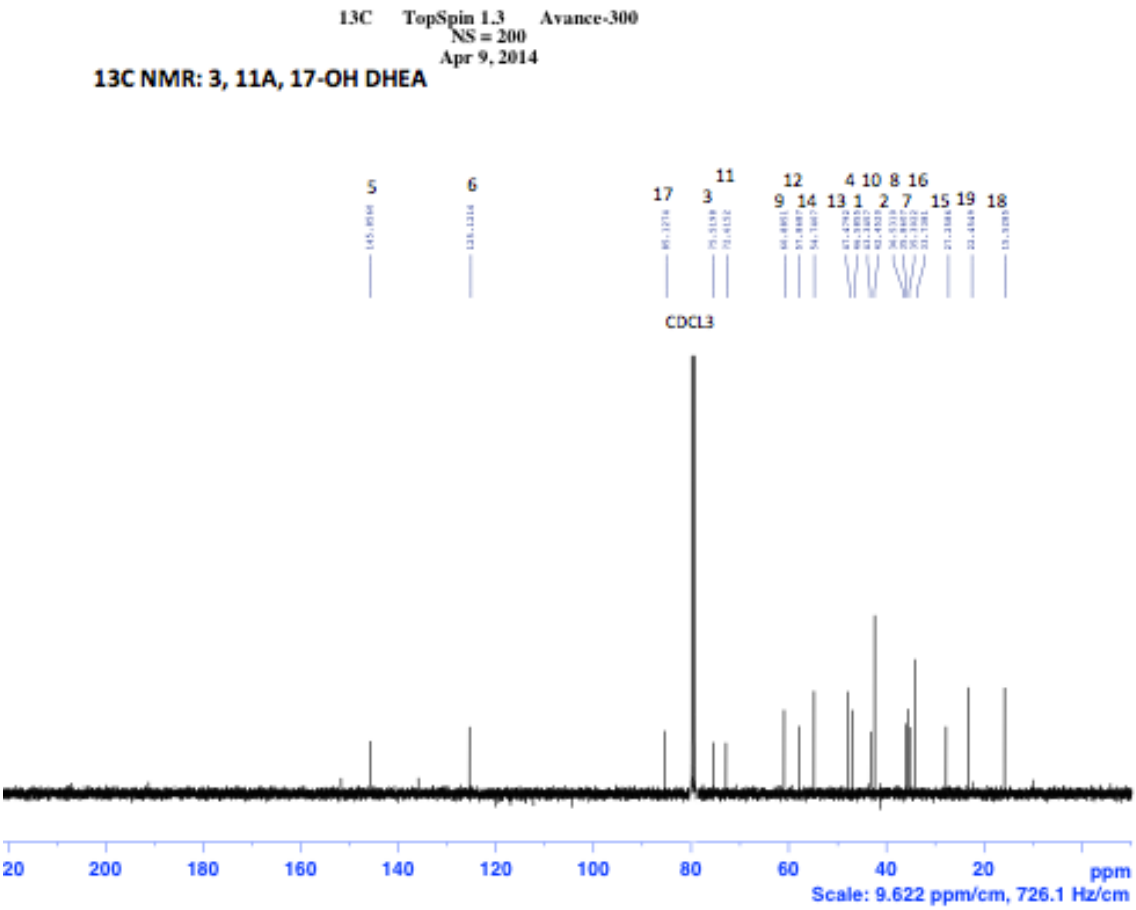


Figure D3: Carbon NMR spectrum of 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

APPENDIX E. QUALITATIVE OBSERVATION OF CELLS ADAPTATION – TRANSMISSION ELECTRON MICROSCOPE

The light microscope was a Olympus BX-51. Each new generation grown at n-alkane was observed on a Transmission Electron Microscope. Results showed a positive adaptation process and remarkable cell morphology changes with each new generation. Cells grown with glucose (previous to n-alkane) have circular shape. After various generations (15 days growth) on n-alkanes, cells were adapted and a branched-mycelia shape was found.

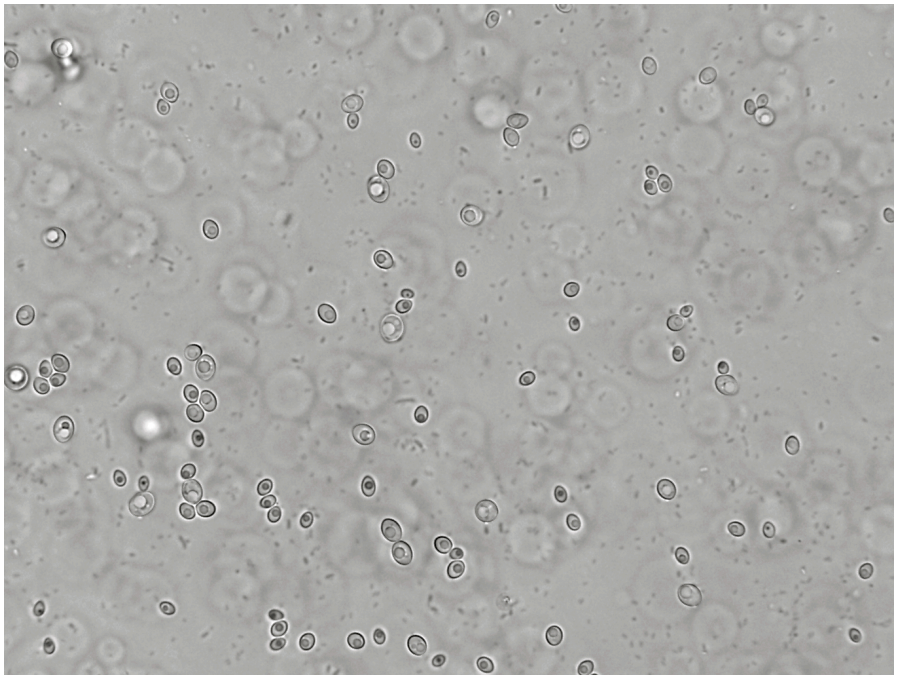


Figure E1: Cells grown with glucose and not exposed to n-alkane (Circular shape.).

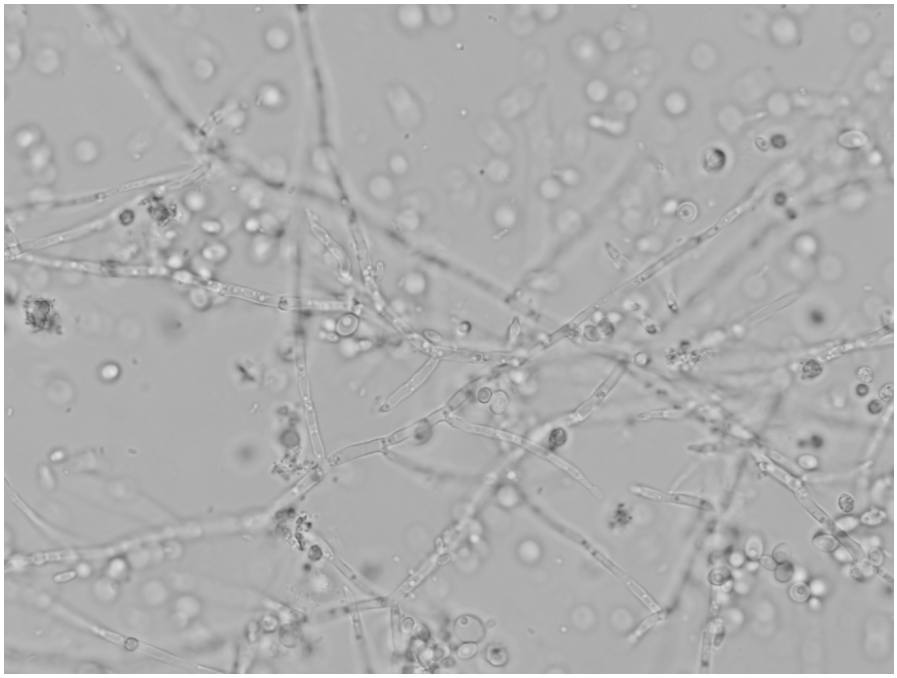


Figure E2: 6th generation of cells grown with n-alkanes (branched-mycelia shape).

APPENDIX F. GROWTH OF MICROORGANISM AND MAINTENANCE OF CELLS CULTURES

Medium A

Dextrose	20 g/L
Yeast extract	5 g/L
Soybean Flour	5 g/L
NaCl	5 g/L
K2HPO4	5 g/L
Deionized Water	1L
Adjust pH to 5 with HCl	

Minimum Medium

K2HPO4	1.0 g/L
MgSO4.7H2O	0.5 g/L
NH4NO3	0.7 g/L
Deionized Water	1L
Adjust pH to 5 with HCl	

CSL Medium (Iowa Medium)

Corn Steep Liquor	20 g/L
Glucose	10 g/L
Deionized Water	1L
Adjust pH to 7	

Potato Dextrose Medium

Diced Potatoes	300 g/L
Glucose	20 g/L

Boil potatoes in 500 m/L of Deionized water. Filtrate, and add water to reach 1L.

Add Glucose before sterilization. pH not needed to be adjusted.

PDB Medium

Following DIFCO instructions. Suspend 24 g of the powder in 1L of deionized water. Heat with frequent agitation and boil for 1 min to completely dissolver the powder. Autoclave at 121°C for 15 minutes. pH not needed to be adjusted.

PDA Medium

Following DIFCO instructions. Suspend 39 g of the powder in 1L of deionized water and boil to dissolve completely. Autoclave at 121°C for 15 mins. pH not needed to be adjusted.

Zhigang Medium

Corn Steep Liquor	20 g/L
Glucose	30 g/L
K2HPO4	1 g/L
MgSO4.7H2O	0.5 g/L
Deionized Water	1L
Adjust pH to 7 with NaOH	

Minimum Medium + Hydrocarbon

Minimum Medium	100 mL
Hydrocarbon	1g (1% W/V) or 10 mL (10% v/v)

Solids hydrocarbons like eicosane, tetracosane, and octacosane, are dissolved in 10mL of hexane.

Buffer

$Na(NH_4)HPO_4 \cdot 4H_2O$ (2.09 g), K_2HPO_4 (1.74 g) per liter of distilled water. The

pH was adjusted to 7. autoclaving for 15 minutes. The solution was allowed to cool down and 5 mL of a 2 M dextrose solution, sterilized by passing it through 0.45 µm filter, was added. 2 M is (3.6 g/10 mL H_2O).

Agar plate preparation

1. Measure the desirable volume of liquid media.
2. Pour it into the serum bottles.
3. Determine the corresponding agar weight to have a 15 g/L concentration.
    a. Agar weight = 15 g/L*(Measured volume)
4. Add the agar into the serum bottles.
5. Autoclave for 10 minutes @ 120°C.
6. Wait for the media agar to cool down.
7. Clean the Biosafety Cabinet.
8. Spray 70% EtOH Solution over each material that is entering the biosafety cabinet.
9. Place the petri dishes cap side up and gently pour agar into them to avoid bubbles formation.
10. Wait at least 1 hour for the plates to cool down.
11. Your plates are ready to use.

Note: Agar starts to gel at 40°C, so make sure to pour it before it reaches that temperature. If bubbles are formed during the pouring process, you can stir the plate in a 8 form motion or gently beat it (to avoid splashing agar over the cap). Be careful in the amount of agar you used. If you used too much is harder to pour into the petri dishes and might affect cell growth. The preferable concentration range is between 15 g/L to 30 g/L and it depends on the media that you are using and the cell you are trying to grow.

Preparation of Cell frozen stock

1. Place a couple of cap open 2 mL vials into a 250 mL beaker.
2. Autoclave your pipette tips, vials, tweezers, and glycerol solution for 10 min at 120°C.
3. Clean the biosafety cabinet with the 70% EtOH solution.

4. Place the vial into the vial holder, cap closed.
5. Pipet 500 µl of 30% glycerol solution into each vial, this is for shorter storage.
6. Stir the cell broth flask.
7. Take the Styrofoam stopper with your tweezers, never with your hands.
8. Pipet 500 µl of cell broth into each vial.
9. Check that each vial is half full before next step.
10. Label them with your initials, date and strain number.
11. Transfer the vials into a cryogenic vial holder, which should be labeled with the microorganism name and your initials.
12. Store them at -80°C ultra-freezer.

Note: For longer storage the final glycerol concentration should be between 25-30% in order to guarantee longer cell viability.

APPENDIX G. PRELIMINARY SCREENING OF REACTION PROGRESS

Thin Layer Chromatography (TLC)

5 x 1.5 cm silica coated plates were used with three spots (substrate, reaction, co-spotting).

Revealing Agents: Phosphomolybdic Acid green spot for every substrate and very sensitive to small amounts ; Vanillin Stain; different colors depending on the products functional groups and limit to 50 µg. and Acidic EtOH special for steroids.

UV/Vis Spectrophotometry

1 mL cuvettes with reaction broth or organic phase (ethyl acetate extraction), wavelength tune to the major absorbance peak of the substrate (distinguish by running a complete UV/Vis spectrum run and observing where the major absorbance occurs)

APPENDIX H. CONVERSION OF DHEA WITH N-HEXADECANE ADAPTED AND NON-ADAPTED CELLS

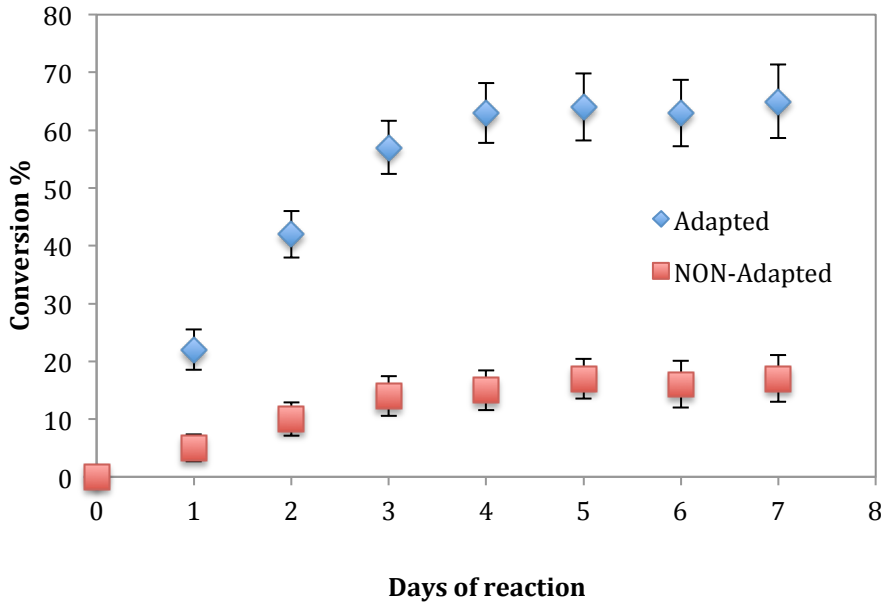


Figure H1: After 7 days of Biotransformation, the conversion of DHEA was higher (70%) with cells adapted to n-alkanes than with non-adapted cells (15%). Results are means ± Std Dev., n = 3.

APPENDIX I. RATIO OF VOLUME (CELLS GROWN ON GLYCEROL TO RESUSPENDED ON BUFFER) EFFECT ON BIOTRANSFORMATION

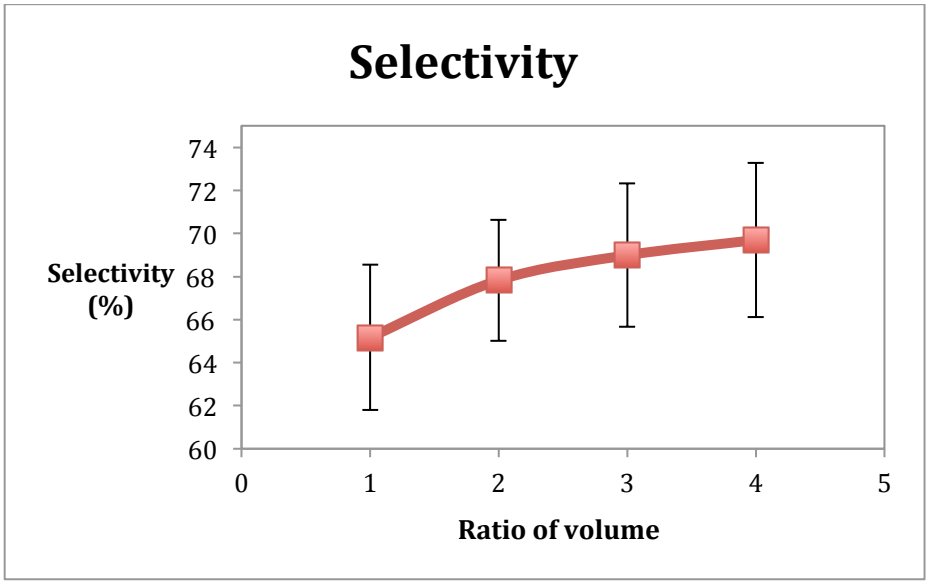


Figure I1: Effect of high volumes of glycerol medium GM compared to buffer solution BS (higher ratios) in biotransformations in the selectivity of desired product. Results are means ± Std Dev., n = 3.

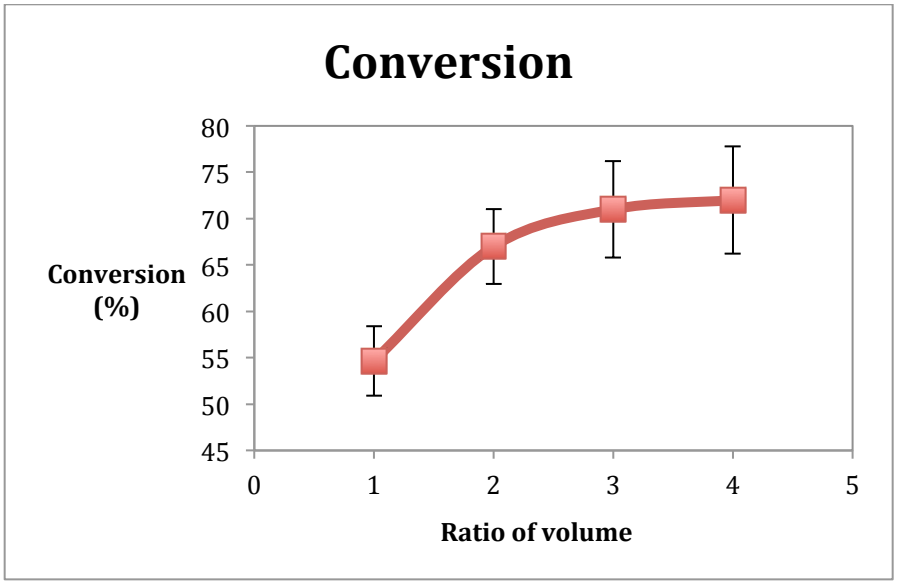


Figure I2: Effect of high volumes of glycerol medium GM compared to buffer solution BS (higher ratios) in biotransformations in the conversion of substrate. Results are means ± Std Dev., n = 3.

APPENDIX J. MICHAELIS-MENTEN KINETICS APPROXIMATIONS

The approximation of these two methods (Lineweaver-Burk plot and Eadie-Hofstee) was used to choose the starting range of K_M and V_{max} values In the iteration of the linear regression in Chapter 3.

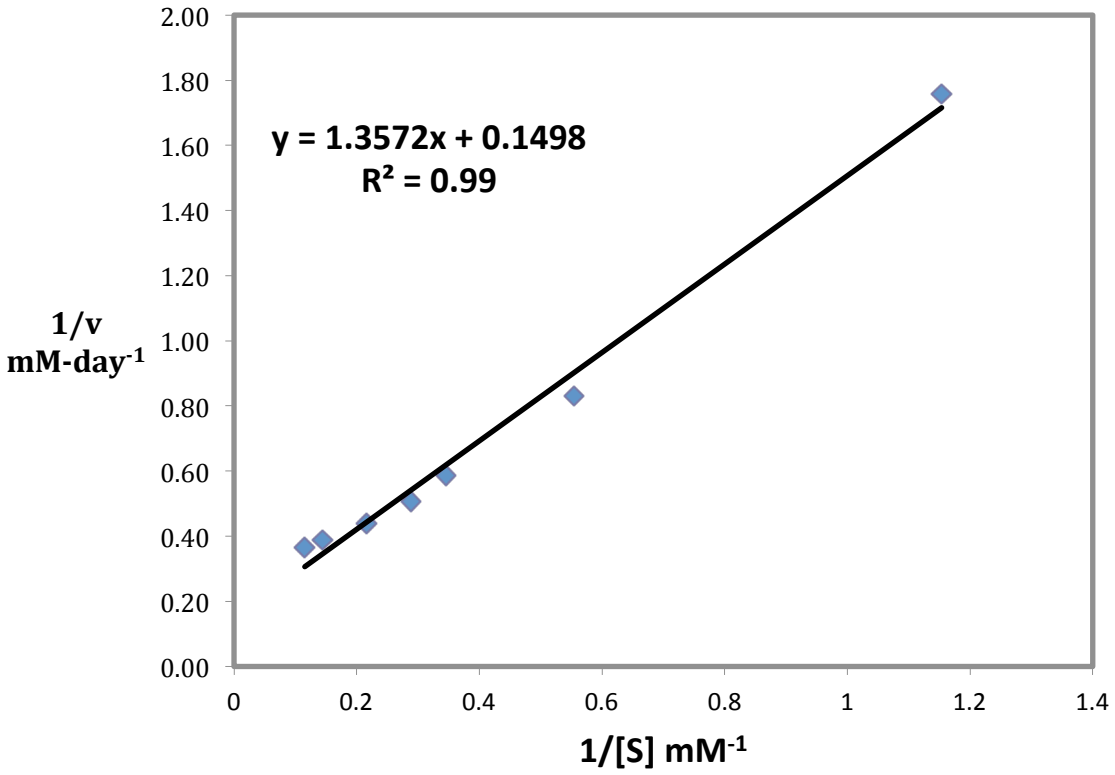


Figure J1: Lineweaver-Burk plot kinetic approach resulted in a K_M of 8.69 mM and V_{max} of 6.43 mM/day.

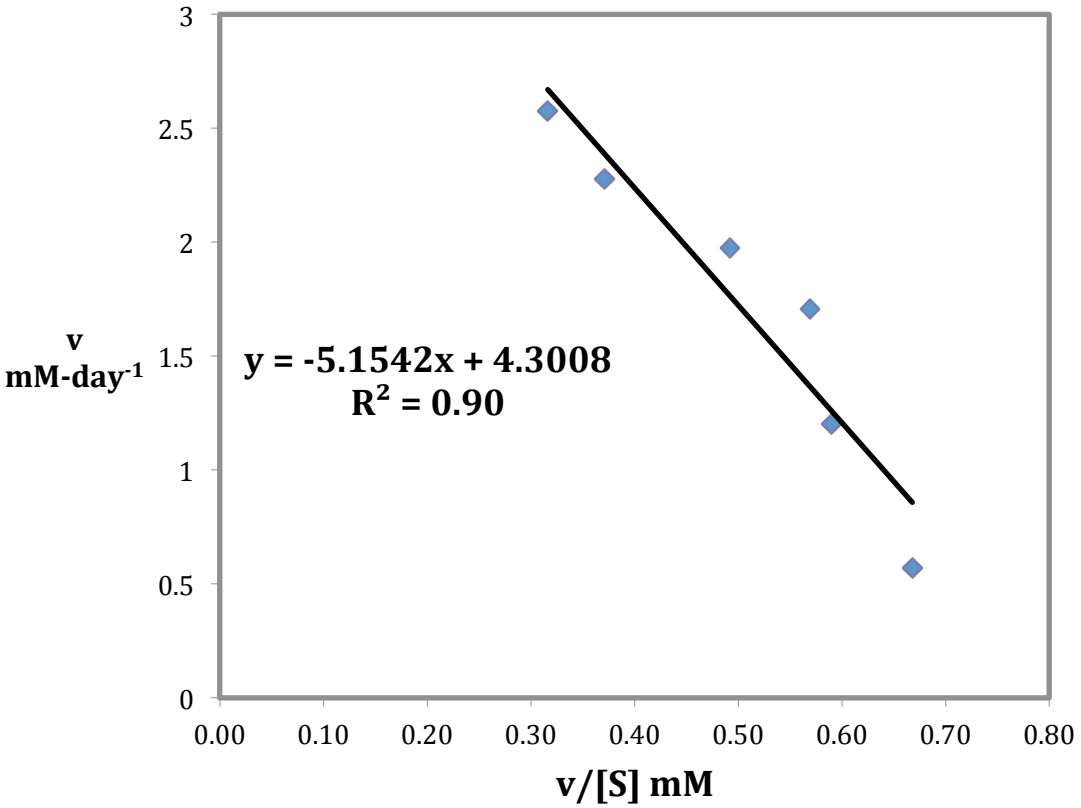


Figure J2: Eadie-Hofstee kinetic approach resulted in a K_M of 4.85 mM and V_{max} of 4.12 mM/day.

APPENDIX K. EFFECT OF ETHNOL AS A SOLVENT IN THE BIOTRANSFORMATION

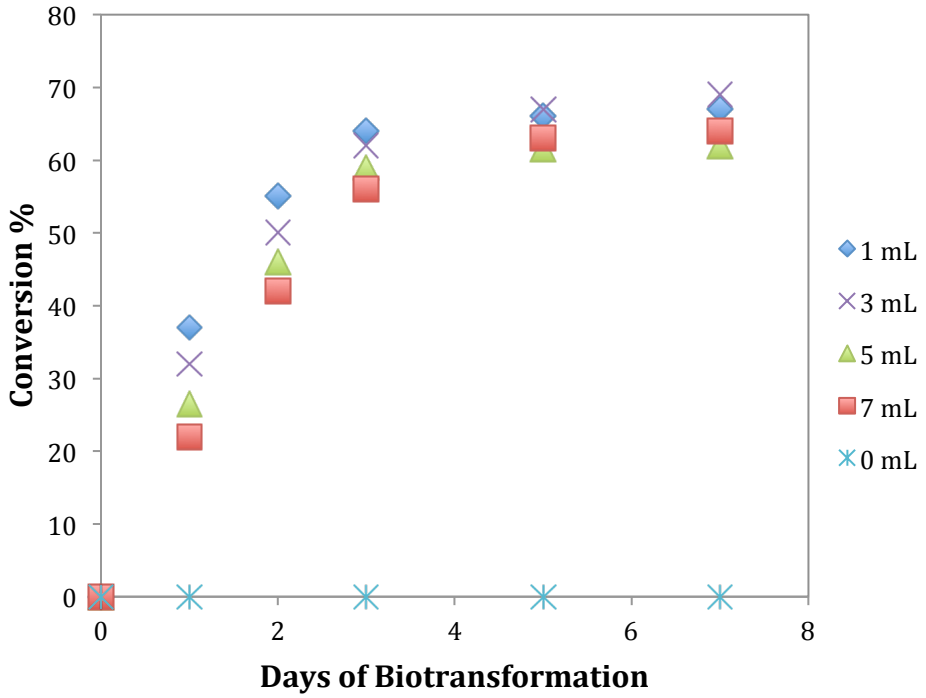


Figure K1: Small volumes of ethanol (1-7 mL) were used to dissolve the substrate DHEA into solution and added to the reaction flask. Ethanol appears to have an inhibitory effect on the reaction, since increasing volumes of ethanol are accompanied by slower conversion of DHEA. After 5 days of reaction, all of the ethanol seems to evaporate and maximum conversion is achieved.

APPENDIX L. CALCULATION OF INTERMEDIATE REACTION KINETICS – ANDROSTENEDIOL (I) AS SUBSTRATE BIOTRANSFORMED TO DESIRED 11-HYDROXY PRODUCT

The velocity or k for this reaction cannot be determined because the dependence of rate on substrate concentration is linear.

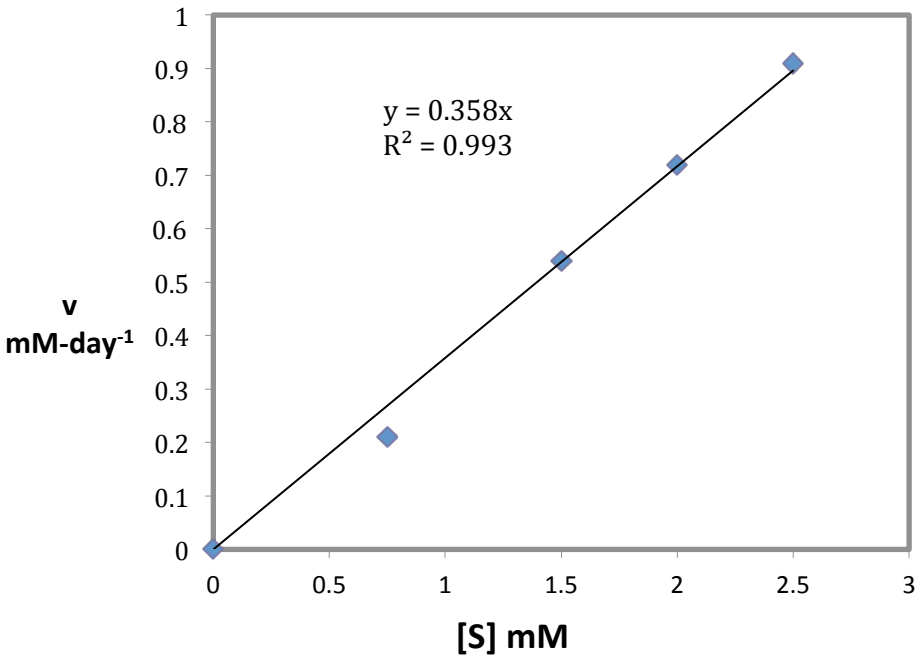


Figure L1: The velocity and reaction constant for the second reaction cannot be determined because the dependence of rate on substrate concentration is linear.

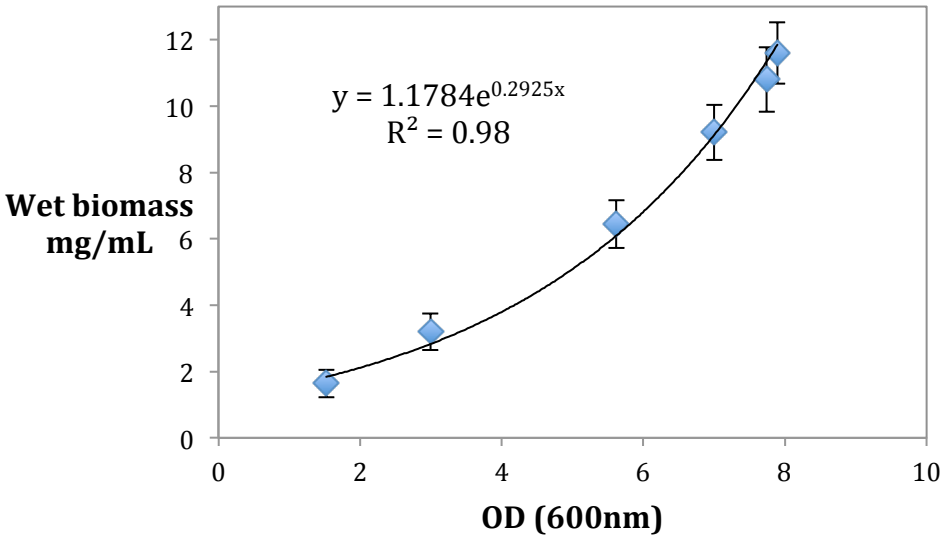


Figure M1: Wet biomass growth of *B. bassiana* during 24 hours as described in Chapter 4. The relation of optical density (analytical equipment) and biomass was used to track the number of cells during the growing and biotransformation stage. Results are means ± Std Dev., n = 3.

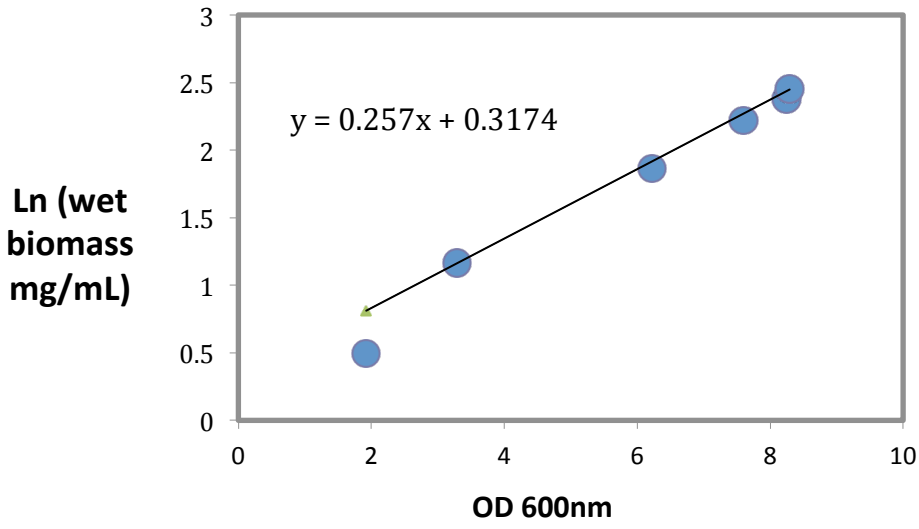


Figure M2: Data from the wet biomass produced on 24 hours growth of *B. bassiana* was linearized to track the number of cells with the spectrophotometer.

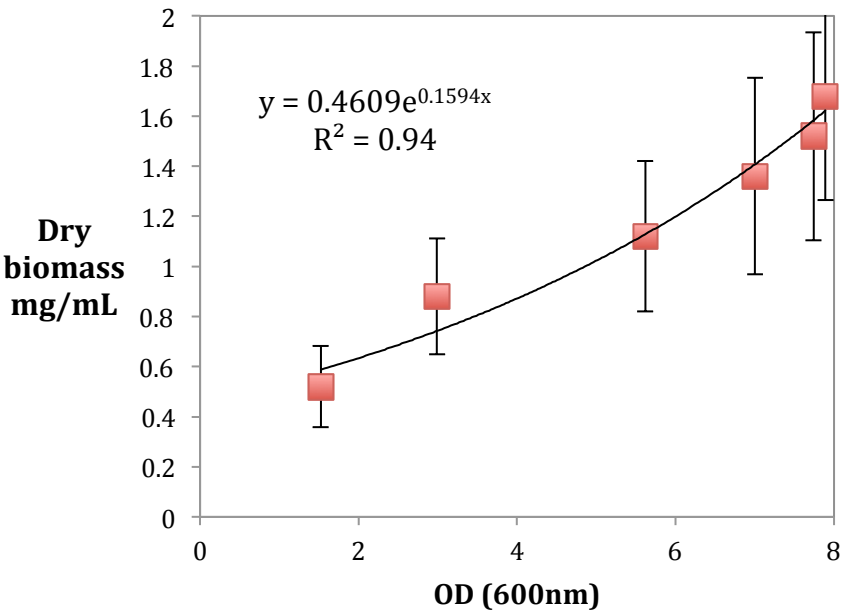


Figure M3: Dry biomass from the growth of *B. bassiana* during 24 hours. Cells were dried for 24 hours in a 100°C oven. Results are means ± Std Dev., n = 3.

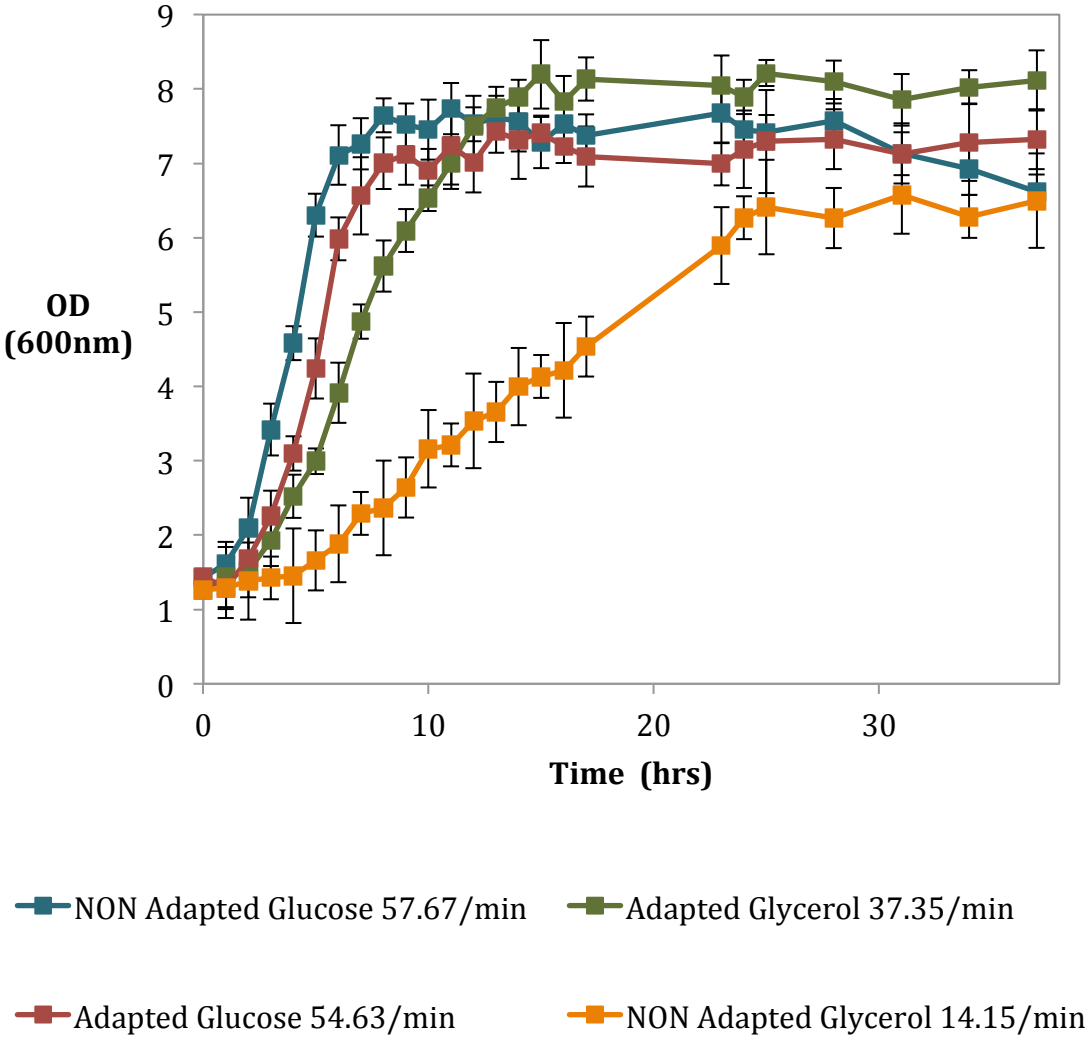


Figure M4: Higher cell density was achieved when 'n-hexadecane adapted cells' inoculated the glycerol culture medium. Results showed that a glucose medium is an efficient carbon source when it comes to faster growth rates. Both adapted and non-adapted cells have the faster growing rate μ (57.67/min and 54.63/min respectively) when grown in glucose. Results are means ± Std Dev., n = 3.

APPENDIX N. EFFECT OF NUMBER OF CELLS ON REACTION YIELD

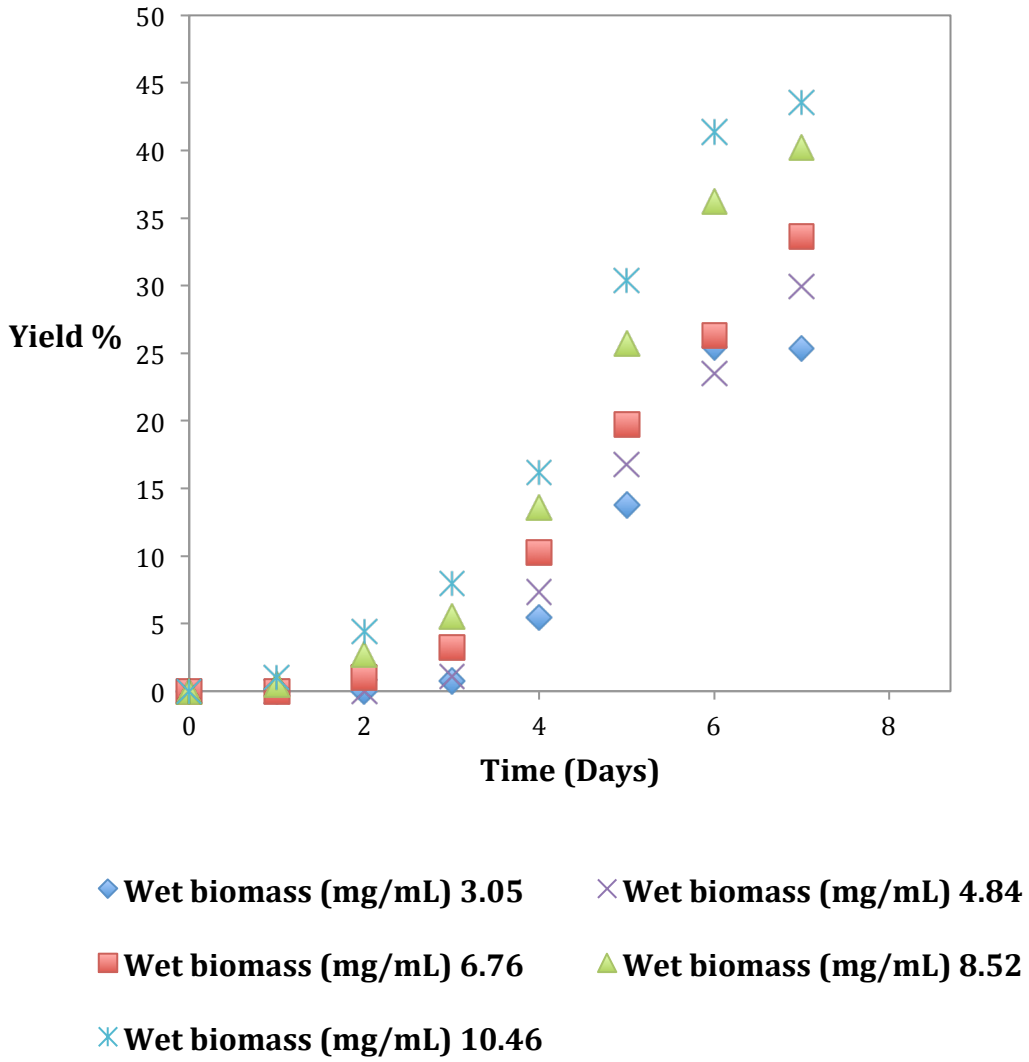


Figure N1: Effect of different biomass concentrations on the reaction yield of desired 3β,11α,17β-trihydroxyandrost-5-ene (**II**).

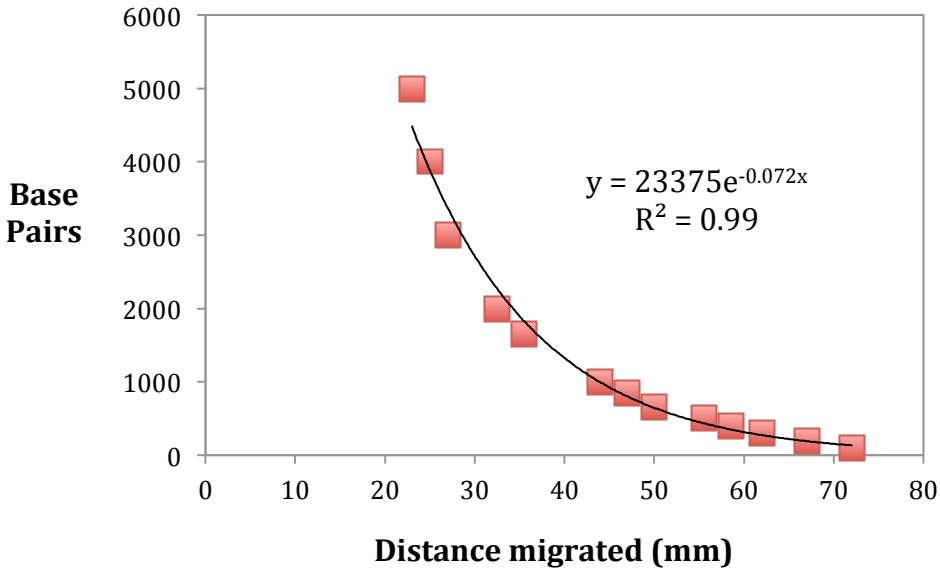


Figure O1: Calibration curve relating distance migrated by DNA band and the number of base pairs in a 1 KB ladder.

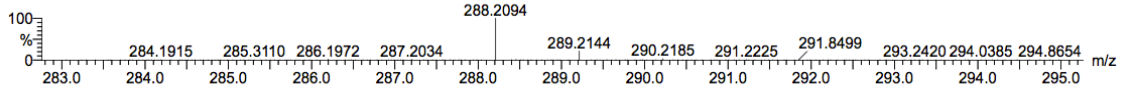
DHEA (Substrate)

Elemental Composition Report **Page 1**

Multiple Mass Analysis: 2 mass(es) processed
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
498 formula(e) evaluated with 4 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
12C: 0-500 13C: 0-1 H: 0-1000 O: 0-5
DHEA 1
G04171412 929 (14.762) Cm (924:929)

TOF MS EI+
6.49e+005



Minimum:	5.00				-1.5					
Maximum:	100.00		5.0	10.0	50.0					
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula			
289.2144	21.10	289.2123	2.1	7.3	6.0	30.9	12C18	13C	H28	O2
		289.2168	-2.4	-8.3	5.5	4304.0	12C19	H29	O2	
288.2094	100.00	288.2089	0.5	1.7	6.0	65.3	12C19	H28	O2	
		288.2045	4.9	17.0	6.5	15131.9	12C18	13C	H27	O2

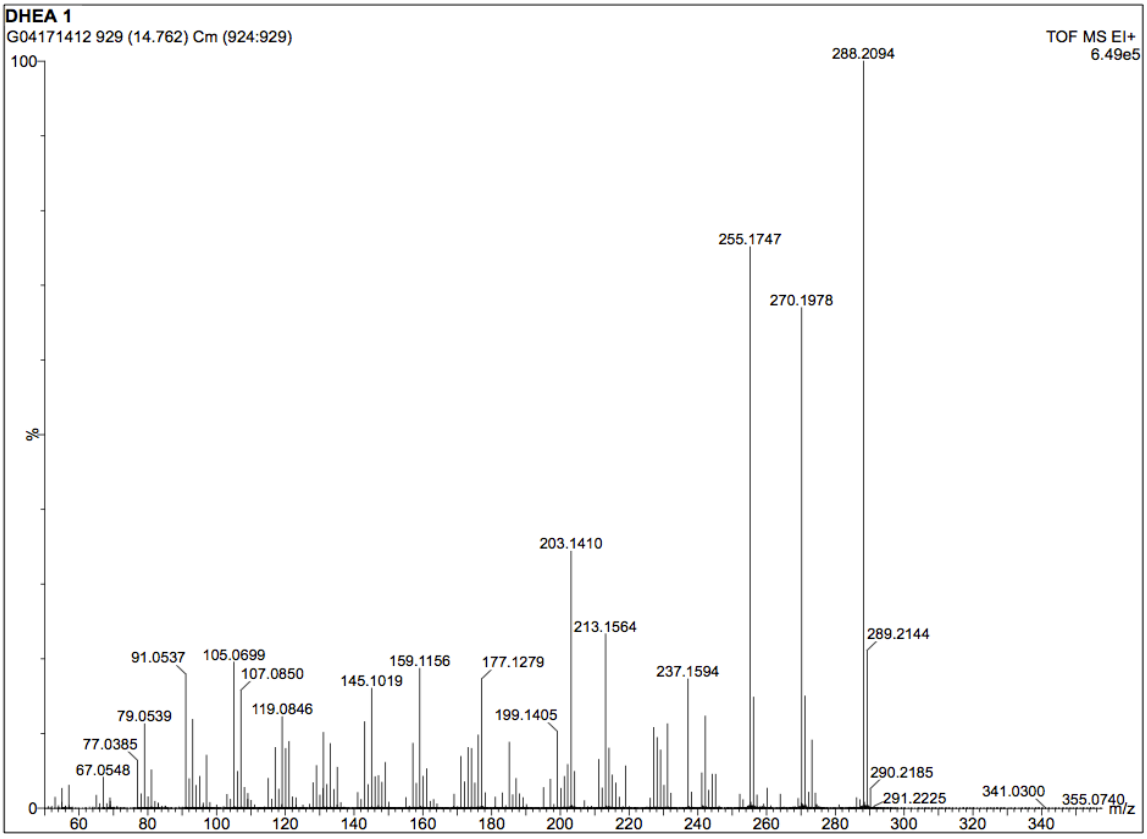


Figure P1: Mass spectrometry analysis shows the molecular weight of DHEA is 289.21 m/z (literature 289.42 g/mol).

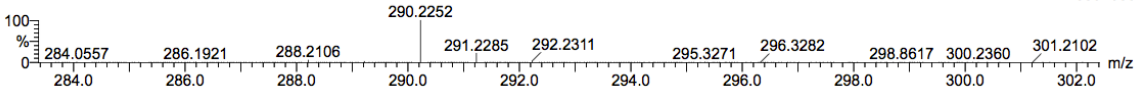
Androstenediol (I)

Elemental Composition Report **Page 1**

Multiple Mass Analysis: 2 mass(es) processed
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
500 formula(e) evaluated with 3 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
12C: 0-500 13C: 0-1 H: 0-1000 O: 0-5
DHEA 2
G04171414 936 (14.837) Cm (929:985-116:454)

TOF MS EI+
4.33e+005



Minimum:	6.00				-1.5					
Maximum:	100.00		5.0	10.0	50.0					
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula			
291.2285	21.39	291.2279	0.6	2.1	5.0	308.9	12C18	13C	H30	O2
		291.2324	-3.9	-13.4	4.5	4106.9	12C19	H31	O2	
290.2252	100.00	290.2246	0.6	2.1	5.0	93.5	12C19	H30	O2	

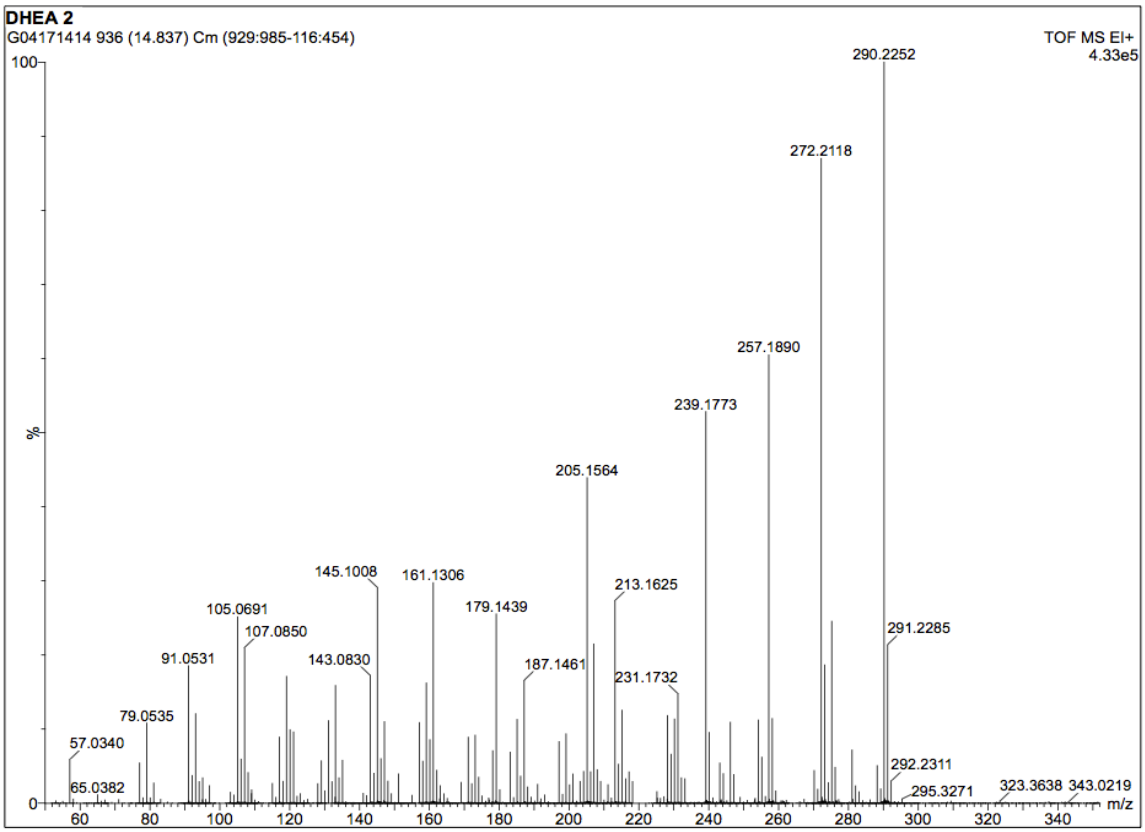


Figure P2: Mass spectrometry analysis shows the molecular weight of intermediate androstenediol (**I**) is 290.22 m/z (literature 290.44 g/mol).

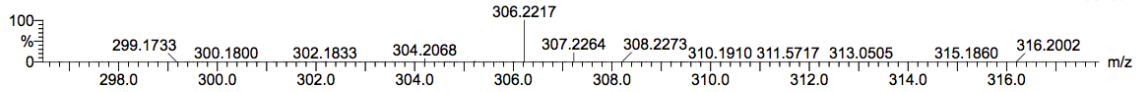
3β,11α,17β-trihydroxyandrost-5-ene (II)

Elemental Composition Report **Page 1**

Multiple Mass Analysis: 2 mass(es) processed
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off

Monoisotopic Mass, Odd and Even Electron Ions
532 formula(e) evaluated with 3 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
12C: 0-500 13C: 0-1 H: 0-1000 O: 0-5
DHEA 3
G04171416 1483 (20.672) Cm (1437:1552-248:726)

TOF MS EI+
7.26e+004



Minimum:	8.00				-1.5					
Maximum:	100.00		5.0	10.0	50.0					
Mass	RA	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula			
307.2264	21.44	307.2228	3.6	11.7	5.0	37.1	12C18	13C	H30	O3
		307.2273	-0.9	-2.9	4.5	705.6	12C19	H31	O3	
306.2217	100.00	306.2195	2.2	7.2	5.0	22.1	12C19	H30	O3	

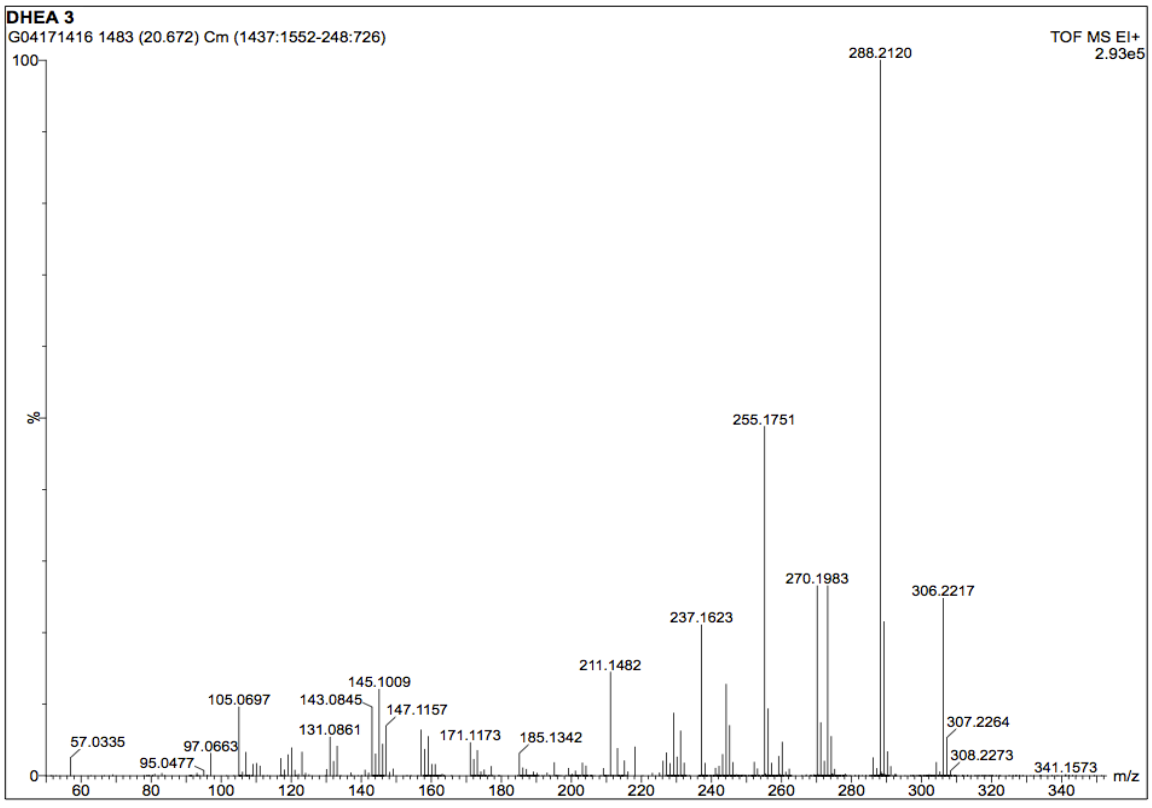


Figure P3: Mass spectrometry analysis shows the molecular weight of desired product 3β,11α,17β-trihydroxyandrost-5-ene (**II**) is 306.22 m/z (literature 306.43 g/mol).

APPENDIX Q. PRIMERS DESIGN FROM IDT

RACE Assay

A. CYP655C1
Name: rp4502f (Forward)
Sequence: CGA CAG CAG CGC ATC AGA GAT GGC CTC C
Name: rp4502r (Reverse)
Sequence: CAT CGC CGA CCG CAT CCA CTT CGT CTC T

B. CYP5337A1
Name: rp4503f (Forward)
Sequence: TTG CTC TCA CCG CAG GTG CCT ACG TT
Name: rp4503r (Reverse)
Sequence: AAC GTA GGC ACC TGC GGT GAG AGC AA

C. CYP617N1
Name: rp4506f (Forward)
Sequence: CGG AGA ATG GCG GCA AGA ATG CGA CCA A
Name: rp4506r (Reverse)
Sequence: CGC ATT GGT CGC ATT CTT GCC GCC ATT C

D. CYP53A26
Name: rp4507f (Forward)
Sequence: CCG GCA TCG CCA TTG CCA GAG TCA AGA G
Name: rp4507r (Reverse)
Sequence: CGA TTC GGC TCT TGA CTC TGG CAA TGG

QRT-PCR Assay

A. CYP655C1
Name: qp4502f (Forward)
Sequence: AAC GAG TAT CGG CCT GAA CGC TG
Name: qp4502r (Reverse)
Sequence: AAT ACG CAT TCT GGC CCT AAA CGG

B. CYP5337A1
Name: qp4503f (Forward)
Sequence: GTC CGG ATG CAA TGG AGT G
Name: qp4503r (Reverse)
Sequence: GCG GCT TAT TAC GAT CGA CC

C. CYP617N1
Name: qp4506f (Forward)
Sequence: GAT GCG CAC GAG TTC AAC C
Name: qp4506r (Reverse)
Sequence: GGA CAT GCC AGC TCC GAT T

D. CYP53A26
Name: qp4507f (Forward)
Sequence: GAG TCA AGA GCC GAA TCG AG
Name: qp4507r (Reverse)
Sequence: CGG CAG AGG TAA TTG AGC AG

APPENDIX R. PCR CYCLING PROGRAM

Step	Time	Temperature (°C)
1. Initial heat activation	15 mins	95
2. **3 step cycling** Denaturation	30 sec	94
3. Annealing	90 sec	57
4. Extension **Go to step 2**	90 sec	72
5. Final extension	10 mins	72

40 cycles.

APPENDIX S. SEQUENCING RESULTS

P-450 gene #2 gene with 250 bp

TNNNNNGTNNCTCCATCGCGCGCNNGGCGATTCTGAGGTCGTCGGCGAAGGCAAGG
GACGGCAAGGAGGCATGTCACCAGGCGCTGGCGCTGGCGCCCGATCTGATCCTGATG
GATCTGTCGATGCGCGGGATGAACGGCATCGATGCGTGCGCAACGATCAAGCGGCG
AATGCCGACGGTCCGGATCGTCGCGCTCACGGTTCACCAGAGACGAAGTGGATGCG
GTCGGCGATGACCNATTTCACCACTTCACTCGCCCTGCCCCCGCCATCCTTGTGGTCT
CGGTGTTATCATCTCTAATCTCCGTGACGGGCTTTTTATGTCTGCTGGCGGATCAATC
TGTATCATATCTTTTGTTTTTGTGGTATCCCAAGGGCCAAAACCCAAAACGAATCTNG
TCGTAGGGACAACTTATCTAGCGCACACGGCTGCTGGAGGGGGCCGCAACTATCATT
TTGGACGGGGGGAGTGCGTGCTGGGAAACGCAGCTAGCTGGTATGGGACCCCGGCA
CCACCCTCAGGTTGGGGGCTTCTAAATGGATGTCTTCCCCGATAGGCACAATCAGTG
TCCGCTCTCACAGCGCTTGTGCCTCTTCCAAGGATGAGCAGATAGAAAACCGAGCCC
TGCTGACTGTTGGGNTCGCTCTCGTTGCTCCTCCCCCTGTGGTGTGGCGTTTGTGTCA
CAGTCTCTACTGACTGCCTCTCGCGGTAGGCGTGCCTGCGAGCCTGCCTAGACACGT
CAGGGAATCTGACGCTACCATATCTCGATGGTGGCTAGCCACGGTATGCTCCATCGG
ATGCTGAGAAGTGTTGACGCATAGCAAGACTTGTGTCACTATCTTGTGNTGTAGTGC
CGGCAGCAGGAGGTGAAACTTTCCCTCTCCTTTTCGTGAAAAAGAGGAAGACGAATG
CTATTGGCTTTTCGTTGTTCCTGAAGAATGGGCCTGGCGGTAATG

NCBI Result:

NUCLEOTIDE BLAST: Search a nucleotide database using a nucleotide query
Query sub range: 50-400
Database: Nucleotide Collection (nr/nt)
Sequence producing significant alignment: Burkholderia cepacia GG4 chromosome 1, complete sequence

B. cepacia is an important human pathogen that most often causes pneumonia in immunocompromised individuals with underlying lung disease.

Identity: 144/161(89%)
Reported at: "Complete Genome Sequence of Burkholderia sp. Strain GG4, a Betaproteobacterium That Reduces 3-Oxo-N-Acylhomoserine Lactones and Produces Different N-Acylhomoserine Lactones"

P-450 gene #2 – with 550 bp

TNNNNNNATCGACGTCGCGATGACGATGATGACGATCCGCACTTGCCGCGGAAATCT
AGGCGCGCGAGCGCGCACTACGCGAGGTGGCGATGAGCTCCTGATGGCCTGTCTGCG
ACGGGACAATGAGGGAGTTGCCTGGCCGCGCATAAAGTTGTTGCTGCCCTGGTCACT
GCCTGCGCGTTCTCGGTTCACCGATGACGAGGGGATGCGGGTTGTCATGAATCTCCG
AGTCGGGACGCAGCGAGGACACCAGCATCCAGTAGAACGGCGCCAGGCAGTAGATG
ACGATGAGGACGATGCCGATGATCGGCAGCACCTTGGCCCAGGTGTTTTTCTTATGG
TGGGTCTCCTGGTCGAAGGTCCCCGGCGCAGTTGCAGTTACGGTGCTCATGACGCCG
CCTCCTTGCTAGCTGCGGCTTGTACAGCATTGGCTTTCTGCGGGGCACGACGTCCACC
CGGACGTCCACCAGAGACGAAGTGGATGCGGTCGGCGATGATCCATAGGTCCTGAT
GGCTTTTGCATTCTCATTCGATCTCCAGCAGCACAATCACTTTGCGTGCCATCCTTGG
GCAGCGCCCCCTACCTCTTCCACGTCCAATACAGTCGCGAGTGCCAGCGTCGAGTTC
GGGGCTCCCTTCGGATCGCTGTCCGCCCAGGTCTCTGCCGGGTTCTGCTCCAGCATCC
GAGAGGTGCGTGGCCTCGGCTGCCGCTCCCTACCTTTGCCTAGCTTGATTTCCCTGGA
GCATTACCTGTTACTTTCTCCCGTGGCCCTCTCGGATAATAGTTTCGCTCCCTGCTGTT
CATGAAGAGTTCGCTGACTGGGGGTCCATAGCTACCTATTACGACAACATGTTCGGT
GCAGTC

NCBI Results:

NUCLEOTIDE BLAST: Search a nucleotide database using a nucleotide query
Query sub range: 50-800
Database: Nucleotide Collection (nr/nt)
Sequence producing significant alignment: Propionibacterium acnes

Propionibacterium acnes is the relatively slow-growing, typically aerotolerant anaerobic, Gram-positive bacterium (rod) linked to the skin condition acne; it can also cause chronic blepharitis and endophthalmitis, the latter particularly following intraocular surgery. The genome of the bacterium has been sequenced and a study has shown several genes can generate enzymes for degrading skin and proteins that may be immunogenic (activating the immune system).

Identity: 393/442(89%)
Reported at: "Complete genome sequence of Propionibacterium acnes type IB strain 6609."

APPENDIX T. PRESENTATIONS AND PUBLICATIONS

Publications

Gonzalez, R.; Nicolau, F.; Peeples T. "n-Alkane Solvent-Enhanced Biotransformation of steroid DHEA by *B. bassiana* as Biocatalyst". Journal of Advances in Biology & Biotechnology 2015: 2(1) 30-37.

Gonzalez, R.; Nicolau, F.; Peeples T. "Optimization of the 11α-Hydroxylation of Steroid DHEA by Solvent-Enhanced *Beauveria bassiana"*. Submitted to Biocatalysis and Biotransformation on January 2015.

Gonzalez, R.; Peeples T. "Biomass Correlations with Enhancement of DHEA Biotransformation". Peer review.

Conference Presentations

2014

González Richard (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the American Institute of Chemical Engineers Annual Meeting, Atlanta, GA. 2014.

González Richard (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the 2013 SHPE national conference, Detroit, MI.

González Richard (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst Poster presented at the 2013 NOBCChE national conference, New Orleans, LA.

González Richard (speaker), Peeples Tonya. "Optimization of the 11α-Hydroxylation of Steroid DHEA by Solvent-Enhanced Biocatalyst" Presented at The University of Iowa College of Engineering Research Open House 2014.

Allara Chris (Speaker), **Gonzalez Richard**, Peeples Tonya. "Temperature and pH effect on biotransformation of steroid DHEA with *Beauveria bassiana as biocatalyst"* Presented at The University of Iowa College of Engineering Research Open House 2014.

2013

González Richard (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the American Institute of Chemical Engineers Annual Meeting, San Francisco, CA. 2013.

González Richard (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst Poster presented at the 2013 NOBCChE national conference, Indianapolis, IN. –**Best Poster Award.**

González Richard (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the 2013 SHPE national conference, Indianapolis, IN.

González Richard (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of DHEA by *Beauveria bassiana* as biocatalyst" Presented at University of Iowa College of Engineering Research Open House 2013.

2012

Felipe Nicolau-Manterola (speaker), **Richard Gonzalez**, Tonya L. Peeples , and Horacio F. Olivo "*Beauveria bassiana* Oxidative Performance". Presented at the University of Iowa College of Engineering Research Open House 2012.

González Richard (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst Paper presented at the American Institute of Chemical Engineers Annual Meeting, Pittsburgh 2012

González Richard (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Paper presented at the 2012 NOBCChE national conference, Washington DC.

González Richard (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Poster presented at the 2012 SHPE national conference, Dallas, TX.

González Richard (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Poster presented at University of Iowa Center for Biocatalysis and Bioprocessing Conference 2012 –**Best Poster Award.**

González Richard (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Presented at University of Iowa College of Engineering Research Open House 2012.

2011

González Richard, Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced Transformations of Steroids by *Beauveria bassiana*" .Presented at NOBCChE Midwest Regional Conference. Indianapolis. 2011.

González Richard, Peeples Tonya, Olivo Horacio, Nicolau Manterola, F "Steroid Transformation by *Beauveria bassiana*". Presented at the SHPE National Conference. Anaheim. 2011.

González Richard, Peeples Tonya, Olivo Horacio, Nicolau Manterola, F "Solvent-Enhanced Transformations of Steroids by *Beauveria bassiana*" Presented at the AIChE National Conference. Minneapolis. 2011.

González Richard, Peeples Tonya, Olivo Horacio, Nicolau Manterola, F. "Solvent-Enhanced Transformations of Steroids by *Beauveria bassiana*". Presented at the COE Research Open House 2011.

Felipe Nicolau Manterola, **Richard González**, Tonya L. Peeples , Horacio F. Olivo. "Gradual exposure to linear long chain hydrocarbons will enhance the oxidative performance of *Beauveria bassiana*." Presented at the Annual CBB Conference 2011.

APPENDIX U. VITAE

Richard Gonzalez

Department of Chemical & Biochemical Engineering
The University of Iowa, Iowa City, IA 52242
linkedin.com/in/richardgonzalezarroyo
richardgonzalezarroyo@gmail.com
Phone: (787) 594-9179

Professional Preparation

The University of Iowa	Chemical Engineering	Ph.D	2015
The University of Iowa	Chemical Engineering	M.S.	2014
University of Puerto Rico, Mayagüez	Chemical Engineering	B.S.	2010

Research Experience

❖ **The University of Iowa**. Iowa City, IA.

Graduate Research Assistant January 2011-May 2015

Used kinetic, process design and optimization principles to characterize biocatalysts performance for the synthesis of steroids for medical applications. Performed enzyme induction to oxidize hormones into products of interest. Successfully developed parameters to produce biocatalysts using organic solvents and extreme environments. Performed Design of experiments, fermentations, reactor design, catalyst synthesis and characterization, flash column chromatography separations, HPLC quantification, and used NMR and LCMS equipment. Improved yield of valuable hydroxylated steroids from 5 to 45% and established kinetics for pilot plant scale up. Performed data analysis using QI Macros statistics to design, optimize and enhance process operations.

❖ **University of Puerto Rico**. Mayagüez, PR.

Undergraduate Research Assistant August 2009-May 2010

Sponsored by the U.S. Air Force and Department of Energy. Achieved the substantial production of biofuel based on the conversion of Syngas, with copper zinc as a catalyst. Completed with Dr. Jose Colucci, the design, and setup of a "single pass reactor tube".

❖ **University of Puerto Rico**. Mayagüez, PR.

Undergraduate Research Assistant January 2007-December 2007

Sponsored by Wyeth Pharmaceuticals. Synthesized pill tablets enclosing several active ingredients, and evaluated the effect of formulation changes on NIR and RAMAN drug con-tent calibration models.

Industry Experience

❖ **SC Johnson**. Racine, WI.

Intern Research and Development Engineer May 2014–August 2014

Lead the integration of (3) new methods for the kinetic analysis of grease removal for cleaner *Fantastik*. Utilized Design of Experiments, Adhesion Phenomena, Dissolution studies and Abrasion tests on different surfaces, to upgrade current cleaning formula and promote washing performance on greasy soils.

❖ **NASA - Glenn Research Center.** Cleveland, OH.

Intern Bioscience and Technology Engineering May 2010–August 2010

Synthesized 7 new catalysts after extensive literature review, for SYNGAS FT process to produce jet fuel from natural gas. As well, characterization of catalysts with SEM. Results contributed to an increase of biomass production and a publish article at AIAA.

❖ **US ARMY Corps of Engineer** - Champaign, IL.

Intern Materials and Science Engineering May 2009–August 2009

Performed a life cycle assessment, compile analytical data and literature, to write an article about thermoplastic materials for construction applications. Validated the benefits of reducing greenhouse gas emissions using recycled plastic as a replacement for chemically treated wood bridges on Department of Defense installations.

❖ **Eastman Chemical Company** - Kingsport, TN.

Co-op Engineer Jan. 2008–December 2008

Process Engineer:

Worked closely with operators, manufacturing leadership, and fellow engineers to identify improvement opportunities at the 800tons/day TPA-polymer manufacturing process plant. Improved catalyst control scheme by designing a computational algorithm that provides in-situ feedback of reactants. As well, increased reactor pumps capacity by evaluating impellers performance and working with contractors to accomplish impellers replacement.

Research and Development Engineer:

Perform kinetic experiments on a semi-batch reactor to model aldehyde oxidations. Data was analyzed on Dynochem software and resulted on a 2nd order reaction model. Results improve the upscale design of a 4,000L reactor and another Co-op job offer.

Science Related Experience

❖ **University of Iowa**. Iowa City, IA.

Teaching Assistant August 2013-December 2013

Assisted Dr. Ryan Summers in the Introduction to Biochemical Engineering course. Led a 1 hour discussion every week, taught students how to design biochemical reactors and teach laboratory techniques.

- **University of Iowa**. Iowa City, IA.
  Teaching Assistant August 2010-December 2010

Assisted Dr. Eric Nuxoll in the Mass Transfer and Separations course. Taught students how to use computational separation Chemcad software, and to solve homework problems.

- **University of Iowa**. Iowa City, IA.
  Tutor August 2010-Present

Assist students, ages 9-18, with their homework every Tuesday night at MESA tutoring.

Presentations

2014

- **González Richard** (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the American Institute of Chemical Engineers Annual Meeting, Atlanta, GA. 2014.
- **González Richard** (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the 2013 SHPE national conference, Detroit, MI.
- **González Richard** (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst Poster presented at the 2013 NOBCChE national conference, New Orleans, LA.
- **González Richard** (speaker), Peeples Tonya. "Optimization of the 11α-Hydroxylation of Steroid DHEA by Solvent-Enhanced Biocatalyst" Presented at The University of Iowa College of Engineering Research Open House 2014.
- Allara Chris (Speaker), **Gonzalez Richard**, Peeples Tonya. "Temperature and pH effect on biotransformation of steroid DHEA with *Beauveria bassiana as biocatalyst"* Presented at The University of Iowa College of Engineering Research Open House 2014.

2013

- **González Richard** (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the American Institute of Chemical Engineers Annual Meeting, San Francisco, CA. 2013.
- **González Richard** (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst Poster

presented at the 2013 NOBCChE national conference, Indianapolis, IN. –**Best Poster Award.**

- **González Richard** (speaker), Peeples Tonya. "Solvent-Enhanced biotransformations of Steroids by *Beauveria bassiana* as biocatalyst" Paper presented at the 2013 SHPE national conference, Indianapolis, IN.
- **González Richard** (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of DHEA by *Beauveria bassiana* as biocatalyst" Presented at University of Iowa College of Engineering Research Open House 2013.

2012

- Felipe Nicolau-Manterola (speaker), **Richard Gonzalez**, Tonya L. Peeples , and Horacio F. Olivo "*Beauveria bassiana* Oxidative Performance". Presented at the University of Iowa College of Engineering Research Open House 2012.
- **González Richard** (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst Paper presented at the American Institute of Chemical Engineers Annual Meeting, Pittsburgh 2012
- **González Richard** (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Paper presented at the 2012 NOBCChE national conference, Washington DC.
- **González Richard** (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Poster presented at the 2012 SHPE national conference, Dallas, TX.
- **González Richard** (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Poster presented at University of Iowa Center for Biocatalysis and Bioprocessing Conference 2012 –**Best Poster Award.**
- **González Richard** (speaker), Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced biotransformations of Chemicals by *Beauveria bassiana* as biocatalyst". Presented at University of Iowa College of Engineering Research Open House 2012.

2011

- **González Richard**, Peeples Tonya, Olivo Horacio, Nicolau. Manterola, F. "Solvent-Enhanced Transformations of Steroids by *Beauveria bassiana*" .Presented at NOBCChE Midwest Regional Conference. Indianapolis. 2011.
- **González Richard**, Peeples Tonya, Olivo Horacio, Nicolau Manterola, F "Steroid Transformation by *Beauveria bassiana*". Presented at the SHPE National Conference. Anaheim. 2011.
- **González Richard**, Peeples Tonya, Olivo Horacio, Nicolau Manterola, F "Solvent-Enhanced Transformations of Steroids by *Beauveria bassiana*" Presented at the AIChE National Conference. Minneapolis. 2011.

- **González Richard**, Peeples Tonya, Olivo Horacio, Nicolau Manterola, F. "Solvent-Enhanced Transformations of Steroids by *Beauveria bassiana*". Presented at the COE Research Open House 2011.
- **González Richard**, Peeples Tonya, Olivo Horacio, Manterola Nicolau. "Steroid Transformation by *Beauveria bassiana*". Presented at the SHPE National Conference. Anaheim. 2011.
- Ana B. De La Ree, **Richard Gonzalez**, Aloysius F. Hepp ." Fischer-Tropsch Catalyst for Aviation Fuel Production" NASA. 9th International Energy Conversion Engineering Conference (IECEC) sponsored by the American Institute of Aeronautics and Astronautics. San Diego, California. 2011.
- Felipe Nicolau Manterola, **Richard González**, Tonya L. Peeples , Horacio F. Olivo. "Gradual exposure to linear long chain hydrocarbons will enhance the oxidative performance of *Beauveria bassiana*." Presented at the Annual CBB Conference 2011.

Publications

- **Gonzalez, Richard**, De La Ree, Ana. "Fischer-Tropsch Catalyst for Aviation Fuel Production". AAIA 2011: 5740.
- **Gonzalez, Richard**, Nicolau Felipe, Peeples Tonya. "n-Alkane Solvent-Enhanced Biotransformation of steroid DHEA by *B. bassiana* as Biocatalyst". Journal of Advances in Biology & Biotechnology 2015: 2(1) 30-37.
- **Gonzalez, Richard**, Nicolau Felipe, Peeples Tonya. "Optimization of the 11α-Hydroxylation of Steroid DHEA by Solvent-Enhanced *Beauveria bassiana*". Submitted to Journal of Biocatalysis & Biotransformation on January 2015.
- **Gonzalez, Richard**, Peeples, Tonya. "Biomass Correlations with Enhancement of DHEA Biotransformation". Peer review.

Synergistic Activities

- Multi-Ethnic Engineering Students Association (MESA)
  President. 2013-2015
  Iowa City School District Tutor. 2010-Present.
- Society of Hispanic Professional Engineers (SHPE)
  National Graduate Representative 2014-2015
  National Graduate Committee. 2012-2014.
  Treasurer at The University of Iowa Chapter. 2011-2013.
  Member and Certified Leader. 2007-Present.
- Graduate Student Senate at University of Iowa.
  Chemical Engineer Representative. 2012
- Chemical Engineering Graduate Student Association.
  Initiator and President. 2012-2014.
- NOBCChE
  President 2014-Presen.
  Member. 2011-Present.

- Engineering Effort at Rio Grande Inc. (EERGI). Non-profit organization.. PR.
  Founder member and outreach coordinator. 2011-Present.
- ΖΦΒ Fraternity
  Member. 2007-Present.
  Vice-Chancellor at Mayagüez Chapter. 2009.
- Tau Beta Pi Engineering Honor Society. Inducted member. 2009.
- Golden Key International Honor Society. Inducted member. 2009.
- American Institute of Chemical Engineers (AICHE) Member. 2007-Present.

Awards and Honors

2014

- Advancing Science Award, NOBCChE

2013

- Best Poster Award. NOBCChE National Conference, Indianapolis, IN.
- Uncommon Leadership Award "Philip G. Hubbard".
- Advancing Science Award, NOBCChE

2012

- Best Poster Award. Center for Biocatalysis and Bioprocessing Conference
- Scholarship, Professional Training for Minority Graduate Students, Coach
- Advancing Science Award, NOBCChE
- Winner, Socio-Engineer case study, SHPE-NILA Colorado Conference
- Scholarship, Lockheed Martin National Institute for Leadership Advancement
- Distinguished Mentoring Award "Lilia Abron" Ethnic Inclusion
- Vetter Service Award, Department of Chemical and Biochemical Engineering
- Recognized as "Role Model" by Pedro Falú High School Seniors at Puerto Rico

2011

- NASA Student Ambassador

2010

- Graduate Assistance in Areas of National Need (GAANN) Fellowship
- Department of Energy Scholarship for undergraduate research

2007

- Scholarship: Alliance for Minority Participation in undergraduate research

2005

- Recognition in science: Honor Certificate of Natural Science

Collaborators

- Academic Collaborators: Professor Rodolfo Romañach, Chemistry Department, University of Puerto Rico, Mayagüez.
- Industry Collaborators: Dr. RC Ramaswamy, Eastman Chemicals Co. Anthony Scalia, Eastman Chemical Co. Dr. Art Sutton, SC Johnson. John Whitfield.

- Government Collaborators: Dr. Jose Colucci, US Patent & Trademark. Dr. Ana De La Ree, NASA. Dr. Al Hepp, NASA. Richard G. Lampo, US ARMY Corps of Engineers.
- Ph.D. Advisor: Professor Tonya L. Peeples, Department of Chemical and Biochemical Engineering, University of Iowa.

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Yadav S, Tandam N, Kumar K. “Effect Of Different Carbon And Nitrogen Sources On The Biomass Of *Beauveria Bassiana”*. Int J. Advanced Biologial Research (2013): 3 (3), 374.