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N-alkane Solvent-enhanced Biotransformation of Steroid DHEA by *Beauveria bassiana* as Biocatalyst

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Authors' contributions

This work was carried out in collaboration between all authors. Author RG designed the study, performed the analysis of steroids, wrote the protocol and wrote the first draft of the manuscript. Author FN managed the growth of cells in n-alkanes. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The hydroxylation capacity of *Beauveria bassiana* was enhanced with n-alkane solvents, resulting in a selective and eco-friendly scheme for the synthesis of steroids. A biocatalytic system was engineered to augment the 11α-hydroxylation of dehydroepiandrosterone (DHEA) to valuable intermediates. Exposing and inducing cells into n-alkanes improved the synthesis of 11α-hydroxy derivatives.
Methodology and Results: Reactions were carried out with cells grown with n-dodecane (n-C_{12}) and n-hexadecane (n-C_{16}), 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 and NMR analyses. Experiments without the presence of n-alkanes resulted in 17% conversion of DHEA. Isolated products in this case included: Androstenediol (11.8%mM) and 3β,11α,17β-trihydroxyandrost-5-ene (4.78%mM). Results indicate that only the 3,17-hydroxy derivatives of DHEA undergo the 11α-hydroxylation pathway.
Conclusions: The appearance of the products suggests that the reduction of the C-17 ketone of DHEA is preceded by 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 before obtaining a 3β,11α,17β-triol product.

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1. INTRODUCTION

Beauveria bassiana is a whole-cell biocatalyst used to perform transformation of chemicals due to its versatile biocatalytic power and ease of use [1]. The major application of *B. bassiana* is in the pesticide industry [2]. As a result, most work in understanding the complex cellular functions has been in the area of the ability of *B. bassiana* to act as an insecticide. The impact of oxidative biocatalysis enhancement has not been fully explored. 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 [3]. Since this class of enzymes plays an important role in the conversion of xenobiotic compounds to valuable chemical intermediates [4,5], the objective of the current work is 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 [6-10].

Our interest was to engineer a biocatalytic system that enhances the hydroxylation of unfunctionalized carbons during the conversion of steroids to more valuable metabolites. The catalytic activity of *B. bassiana* was screened using dehydroepiandrosterone (DHEA) as substrate. DHEA is a steroid hormone produced by adrenal glands and is the most abundant circulating hormone in humans [11]. This hormone primarily functions as a precursor to more potent androgens such asandrostenedione, testosterone, estrone and estradiol. Previous studies showed that the hydroxylated derivatives of DHEA serve as metabolic intermediates in the biosynthesis of ring-D lactones through Baeyer–Villiger oxidation [12]. As well, literature reports on steroid transformation by *B. bassiana* provide examples of regioselective α-hydroxylation inC-7, C-19 and C-21 onsteroids [13-15]. 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 3β, 11α, 17β-triol product [12]. The results of our current studies provide a potentially new method to synthesize innovative biologically functioning steroids.

2. EXPERIMENTAL

2.1 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).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 Step liquor were purchased from Sigma Aldrich.

2.2 Preparation of N-alkanes Medium (NM)

n-alkanes (NM) medium was prepared by mixing 0.4g KH_2PO_4, 1.4g Na_2HPO_4, 0.6g $MgSO_4.7H_2O$, 1.0g KCl and 0.7g 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: 50mL of n-C_{12} and 50mL of n-C_{16} [16].

2.3 Preparation of Glycerol-corn Steep Liquor Medium (GM)

Glycerol-corn steep liquor medium (GM) was prepared by mixing 20g of corn steep liquor and 10g 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.

2.4 Preparation of Buffer Solution (BS)

The buffer solution was prepared by mixing 2.09g Na $(NH_4)HPO_4 \cdot 4H_2O$ and 1.74g K_2 HPO_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 5mL of a sterile dextrose (2M) solution was added. The dextrose was sterilized by passing it through 0.45µm filter during the addition to the buffer [17].

2.5 Biotransformation with resting cells

2.5.1 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) have grown successfully using n-alkanes as carbon source from June 2011 to September 2013.

2.5.2 Conditions of cultivation and transformation

Phase 1 cultures (40mL inoculum) were washed twice with sterile water and transferred to 400mL 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 5,000 RPM for 10 minutes, washed with BS (3x50mL) and re-suspended in 200mL of BS, 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°Cfor the reaction time indicated.

2.5.3 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 26ºC. A 40mL inoculum from the previous solution was washed twice with sterile water and transferred to 400mL 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 5,000 RPM for 10 minutes, washed with BS (3x50mL) and re-suspended in 200mL of BS, 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.

2.6 Isolation and Identification of Products

After transformation, the BS was centrifuged and steroids were extracted from the liquid supernatant with ethyl acetate (3x50mL). After removal of the solvent, metabolites were analyzed with Thin Layer Chromatography (TLC) and separated by silica gel column chromatography (2cm×10cm) 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 (4.6mm×125mm). Isocratic column elution was monitored by a photodiode array detector. The wavelength was set at 250nm and a methanol–water (60:40, v/v) mobile phase was eluted at a flow rate 0.5mL/min. The ^{1}H NMR Spectra were recorded at room temperature on a 300MHz Bruker Avance spectrometer using deuterated chloroform ($CDCl_3$) as a solvent and Tetramethylsilane as an internal standard.

3. RESULTS

3.1 Products Isolated in the Course of DHEA Transformation

After 7 days of transformation of 200mg of DHEA dissolved in 5mL of ethanol with cells adapted to n-alkanes, 65%±6.3 of the substrate was metabolized. Fig. 1 shows the structure of the biotransformation products. Isolated products included (Average mg± Standard Deviation, % mol): androstenediol (**I**) (81.25±12.7mg, 40.3%) and 3β, 11α, 17β-trihydroxyandrost-5-ene (**II**) (48.65±6.9mg, 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±3.46mg, 11.8%) and **II** (10.2±6.3mg, 4.78%). Experiments were performed in triplicates.

3.2 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 3.64ppm (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 3.93ppm (dt) consistent with substitution at anequatorial proton. Downfield β-carbon shifts in the ^{13}C NMR spectra for C-9 (Δ 7.9ppm) and C-12 (Δ 18.4ppm) confirmed hydroxylation at C-11 (see Table 1).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; HPLC *Rt:* 5.23 min.

3β, 11α, 17β-trihydroxyandrost-5-ene (**II**) GCMS 306.22m/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.

3.3 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. Data for the time course of experiments is shown in Table 2. 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.

Results indicated that enzymes catalyzing the hydroxylation reaction might be 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.**

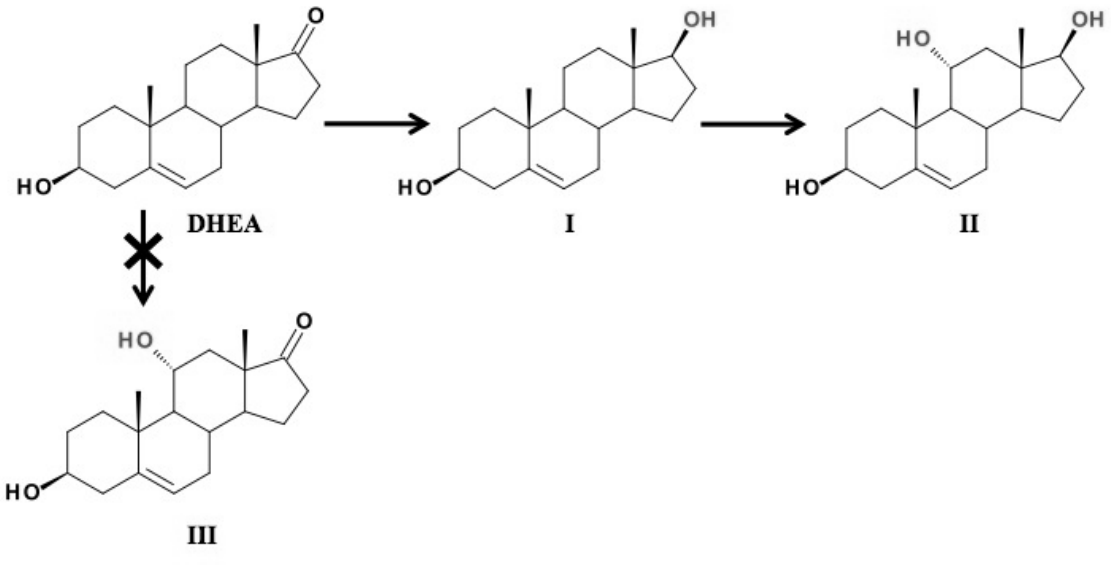

Fig. 1. DHEA to 11α-hydroxylation pathway. After 7 days DHEA biotransformation, there was isolated (% mol): Androstenediol (I) (40.3%) and 3β, 11α, 17β-trihydroxyandrost-5-ene (II) (22.8%). No 11α-Hydroxy-DHEA (III) was synthesized

Table 1.^{13}C NMR data for starting material DHEA and metabolites androstenediol (I) and 3β, 11α, 17β-trihydroxyandrost-5-ene (II) determined in $CDCl_3$

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

Table 2. 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

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-hydroxyderivatives 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.

4. 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-hydroxy steroid. Analysis of composition of the product mixtures as function of reaction time indicates that only the 3,17-hydroxy derivatives of DHEA undergo the 11α-hydroxylation. After 4 days transformation, the reaction mixture contained 3, 17-hydroxy 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-triolproduct (see Fig. 1) 11.

The observed differences in product selectivity and catalytic activity of strain 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 inductors of Steroid-11α-hydroxylase (see Fig. 2). The selectivity of 11α-hydroxy (ratio of **II** and 3-hydroxy-17-oxo derivatives**)** increased from 41% to 60% and the cultures produced 19.27mg more of desired product, just by growing the inoculum of cells with n-alkanes as the carbon source.

It seems that the oxidation of the ketone to17β-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 substratesimplicates several continuous transformations and involves an intensive gene expression ofenzymes like alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthase, to provide the desired fatty acyl-CoA for the β-oxidation metabolic pathway [18,19]. 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.

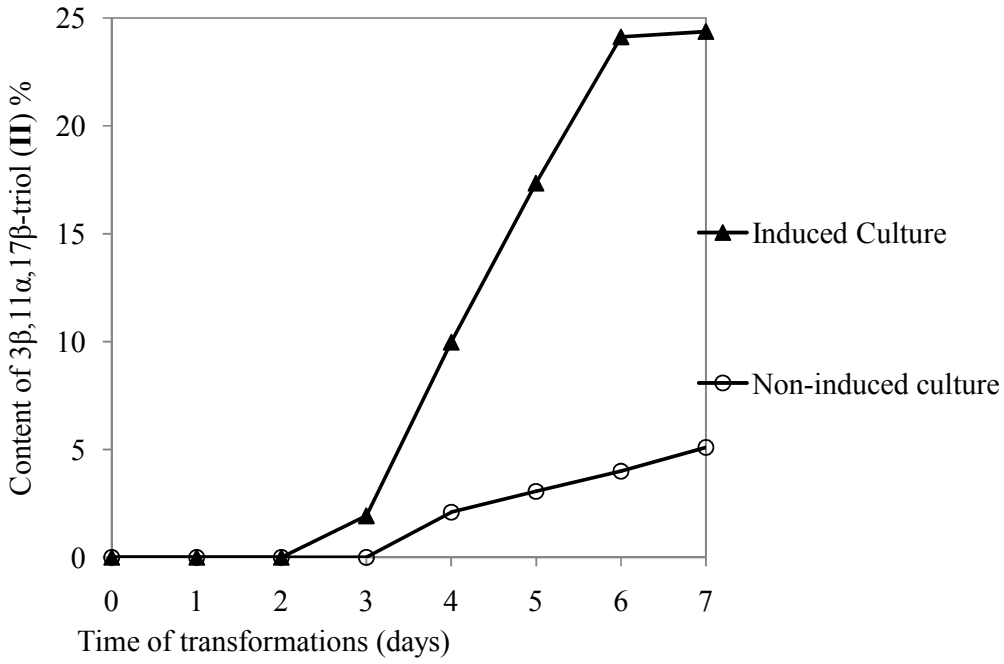

Fig. 2.Comparison of percentage of 3β, 11α, 17β-trihydroxyandrost-5-ene (II) in the mixtures after transformation of DHEA by the non-induced and n-alkane-induced cultures of *Beauveria bassiana*

5. 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 is preceded by 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**.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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