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"Two-step Enzymatic transformation of Grape Seed Oil to Produce a New Hair Growth-Stimulating active ingredient"

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1. Introduction

The demand for effective and sustainable hair growth solutions has been increasing globally. Hair loss affects a significant portion of the adult population, with estimates ranging from 60-70% worldwide [1]. In China, for example, the prevalence of hair loss is approximately 21.3% for men and 6% for women [2]. Hair loss can lead to psychological issues such as anxiety, depression, and low self-esteem, impacting social interactions and professional lives [3]. Therefore, early intervention and prevention of hair loss are crucial [3].

Traditional methods for treating hair loss include synthetic chemicals [4] (minoxidil, finasteride), hair transplantation, and low-level laser therapy. However, these treatments often present challenges such as limited efficacy, time consumption, and side effects. Consequently, there is a growing need for safer and more efficient therapeutic alternatives.

Phytochemicals have emerged as potential agents for managing hair loss due to their growth-stimulating properties. These natural compounds can modulate various biological pathways to promote hair health and regeneration [5].

Castor oil has been traditionally used for centuries to promote hair growth, despite the limited scientific data supporting these claims. This vegetable oil, extracted from the seeds of the Ricinus communis plant, is particularly rich in ricinoleic acid, a hydroxylated fatty acid that constitutes about 85-95% of its composition. Ricinoleic acid is unique due to its hydroxyl group on the 12th carbon atom, which imparts specific properties to the oil. Although the exact mechanisms by which castor oil may enhance hair growth are not fully understood, its traditional use persists, bolstered by anecdotal evidence and its inclusion in numerous hair care products.

Prostaglandins are lipid compounds derived from arachidonic acid, a polyunsaturated omega-6 fatty acid, through the cyclooxygenase (COX) pathway. These compounds play a crucial role in various physiological processes, including inflammation, vascular tone, and hair growth. Specifically, prostaglandins such as PGF2α and PGE2 have been shown to stimulate hair growth by prolonging the anagen (growth) phase of the hair cycle. Conversely, PGD2 has been found to inhibit hair growth [6]. Growth factors like VEGF also play a crucial role on the proliferation of hair follicle dermal papilla cells [7]. Given the importance of prostaglandins in hair follicle regulation, enzymatically modifying fatty acids from vegetable oils to produce molecules with similar structures could potentially mimic the hair growth-stimulating effects of prostaglandins [8].

This study aims to develop a bioactive ingredient derived from grape seed oil through a green enzymatic process to stimulate hair growth. The process involves a two-step enzymatic method: first, the hydrolysis of grape seed oil to release free fatty acids, followed by the specific hydroxylation of linoleic acid. This approach, combining the richness of beneficial fatty acids in grape seed oil with enzymatic biocatalysis, offers a sustainable and effective solution for promoting hair growth.

2. Materials and Methods

2.1. Production of enzymes

In this study, enzymes are used as wet biomass. The hydratases and lipase are selected from Protéus by SEQENS collection and were expressed in *Escherichia coli*. Theire sequence have been optimized for the expression host, and the genes encoding the enzymes have been synthetized by Eurofins. Cloning was carried out in the pET26b+ vector (Novagen) with the *E. coli* BL21DE3 strain as expression host. For lab trials, cultures were grown in autoinducer medium at 37 °C for 4h, then at 20 °C for 20h. Cultures were centrifuged to recover the wet biomass.
Tween 40, Linoleic acid >95% were purchased from Sigma and the oleic acid was purchased from TCI. Novozyme® 435 was purchased from Novozymes (equivalent of Sustine® 110 IM from Novonesis).

2.2. Screening assay

Screening conditions for fatty acid hydroxylation have been performed as follow to determine the kinetics of each enzyme on the commercial substrates of linoleic acid and oleic acid. A 100 mM citrate/phosphate buffer at pH 6.5 is prepared containing 0.5% vol of Tween 40. Stock solutions of enzymes are prepared in the buffer at 85 g/L. A precise volume of commercial acid substrate is added to each vial to reach 30 g/L in oil. Buffer and enzymatic solution are added to reaction vials containing the acids in equal volumes. The reactions are maintained at 40 °C under stirring and the conversion rate is measured during 4h by GC-FID monitoring.

2.3. Analytical method

The samples of the reaction are acidified to pH 1 with 37% HCl and extracted with 2 volumes of dichloromethane. The media is centrifuged to dilute the lower organic phase in a derivation mix of Pyridine/HMDS/TFA 1:1:0,04 vol to reach the theoretical concentration of 1 g/L. The samples are incubated during 1h at 70 °C before GC-FID analysis.
This sample preparation can be applied to characterization analysis in GC-MS performed on a VF-5ms + EZ-Guard (30 m x 0.25 mm x 0.25 µm) column purchased from Agilent or for conversion monitoring in GC-FID performed on a ZB-5HT Inferno (20 m x 180 µm x 0.18 µm) purchased from Phenomenex.

2.4 Prostaglandins and VEGF secretion assay

Dermal papilla cells from hair follicles are cultured on a 6-well plate at 37°C, 5% CO2 for 24 hours before treatment with the four prototypes. 48 hours after treatment, the culture supernatants are collected. The amounts of secreted prostaglandins E2 and D2 are measured using ELISA assays (PGE2 ELISA Kit, Enzo Life Sciences; Prostaglandin D2-Mox ELISA Kit, Cayman Chemicals; Human VEGF SimpleStep ELISA Kit, Abcam) and normalized by cell viability.

3. Results

3.1. Enzyme selection :

In the first phase, to achieve proof of concept, a selection of native enzymes was carried out, these biocatalysts are produced by expression in *E. coli*. 10-hydratases, 13-hydratases and 10,13-hydratates were selected to evaluate their ability to hydroxylate two model substrates: oleic acid and linoleic acid. The expected products are shown in Figure 1.

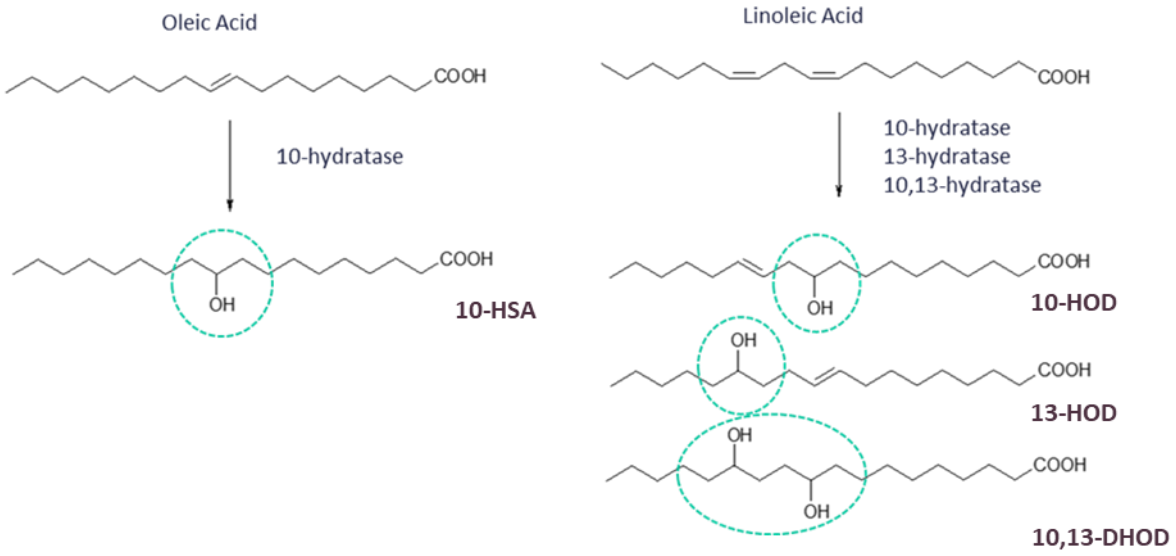

Figure 1. Hydroxylation strategy of model substrates: oleic acid and linoleic acid (10-HAS : 10-hydroxystearic acid; 10-HOD : 10-hydroxy-12-octadecenoïc acid; 13-HOD : 13-hydroxy-9-octadecenoic acid; 10,13-DHOD : 10,13-dihydroxystearic acid)

8 10-hydratases were tested on oleic acid. As shown in Figure 2, only 4 of them achieve maximum oleic acid conversion in less than one hour.

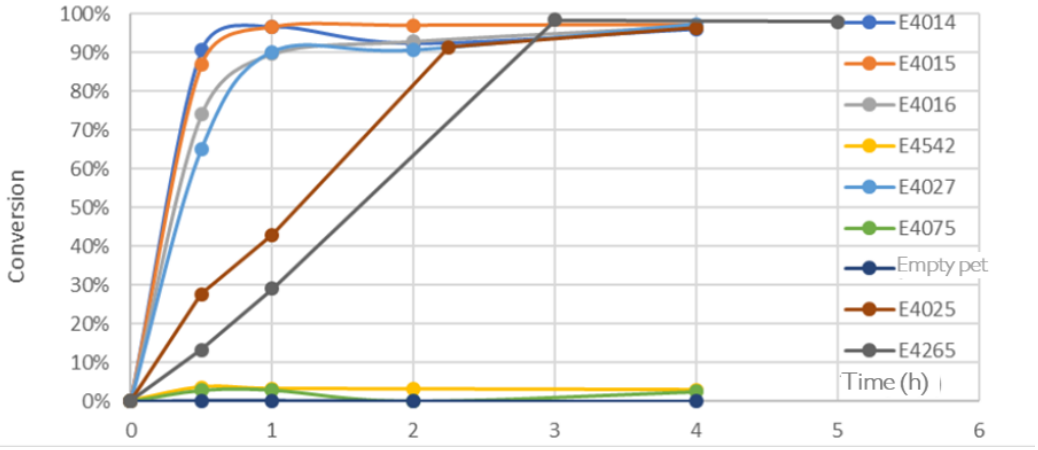

Figure 2. Evolution of oleic acid conversion over time using 8 10-hydratases.

For linoleic acid, in addition to the 8 10-hydratases tested on oleic acid, a 13-hydratase and a 10,13-hydratase were tested. Figure 3 shows the activity of 10-hydratase E4014, which shows a conversion plate of 20% after 1 hour, while the other 10-hydratases do not exceed 10% conversion. The activity of 13-hydratase E4026 allows 54% conversion to be reached in 4 hours without reaching a plateau. Finally, the 10,13-hydratase enzyme E4074 shows no activity on linoleic acid.

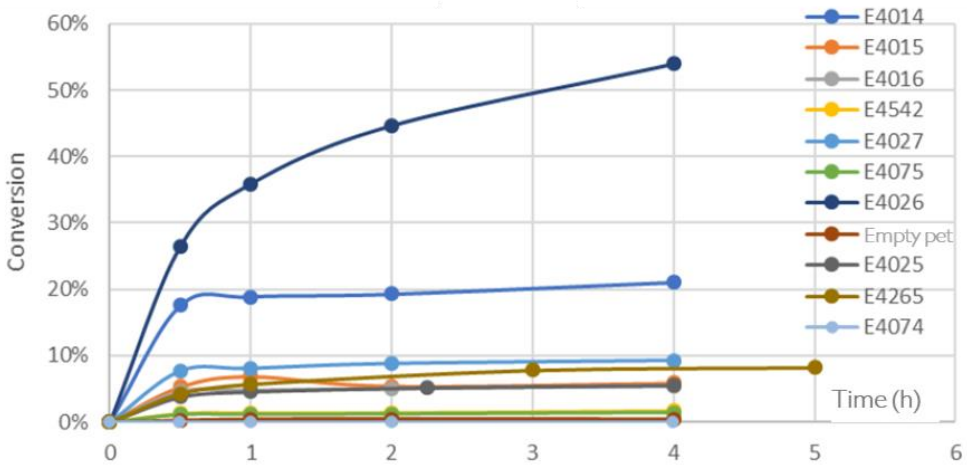

Figure 3. Evolution of linoleic acid conversion over time using 8 10-hydratases, one 13-hydratase and one 10,13-hydratase.

In order to produce four different prototypes of hydroxylated free fatty acids, we selected E4014 10-hydratase and E4026 13-hydratase. Different parameters are modulated in order to optimize reaction productivity: salinity, cofactors & cofactors recycling system, pH, temperature. The 4 prototypes were obtained with a minimum of 70% conversion and 10-HAS, 10-HOD, 13-HOD, and 10,13-DHOD structures were confirmed by GC-MS.

Prototypes were evaluated through an *in vitro* model of Hair Follicle Dermal Papilla Cells on PGE2, PGD2 and VEGF synthesis stimulation and this is the prototype 13-HOD that shows the most promising results leading to the selection of the 13-hydratase E4026 for the two-step biotransformation of grape seed oil.

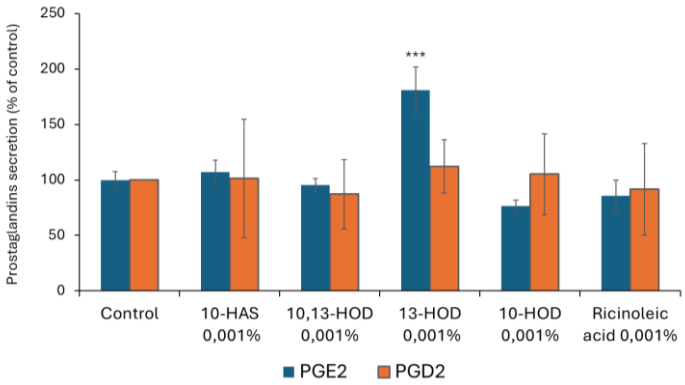

Figure 4. Effect of the different prototypes of hydroxylated free fatty acids and of ricinoleic acid on Prostaglandins E2 (PGE2) and D2 (PGD2) secretion by fair folicle dermal papilla cells. Statistical Student's t-test: *** $p<0.001$ versus control.

As shown in Figure 4, 13-HOD is the only prototype significantly stimulating PGE2 secretion (+80% at 0.001% and +193% at 0.0005%) without any effect on PGD2.

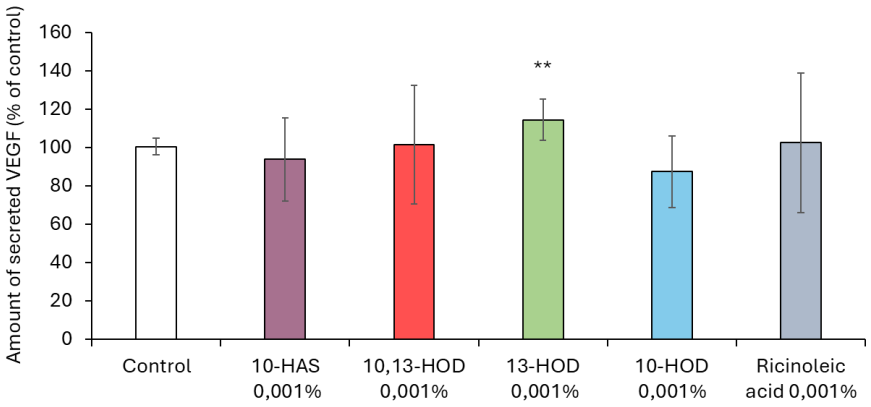

Figure 5. Effect of the different prototypes of hydroxylated free fatty acids and of ricinoleic acid on VEGF secretion by fair folicle dermal papilla cells. Statistical Student's t-test: ** p<0.01 *** p<0.001 versus control.

As shown in Figure 5, 13-HOD is the only prototype significantly stimulating VEGF secretion (+15% at 0.001%). Ricinoleic acid, used as reference, has no effect on both prostaglandins and VEGF secretion.

3.2. Grape seed oil two-step biotransformation:

In order to release free fatty acids from grape seed oil, two lipases were evaluated, a commercial one Novozym435 and a Proteus lipase E4375. The greatest conversion rate with a minimum of 95% was obtained with E4375. Optimal hydrolysis conditions were found to be at a maximum of 60% vol of grape seed oil in buffered solution and 50mg/mL of native enzyme. Subsequently to the hydrolysis and without purification step, the conversion of linoleic acid was targeted with E4026 to obtain 13-HOD with 70% conversion. After scale-up steps and a final purification step with ethyl acetate extraction, we were able to obtain an active titrated at 50% minimum in 13-HOD. Other fatty acids detected are represented by linoleic, oleic, palmitic and stearic acids, main fatty acids present in grape seed oil. Figure 4 summarizes the two-step biotransformation performed on grape seed oil.

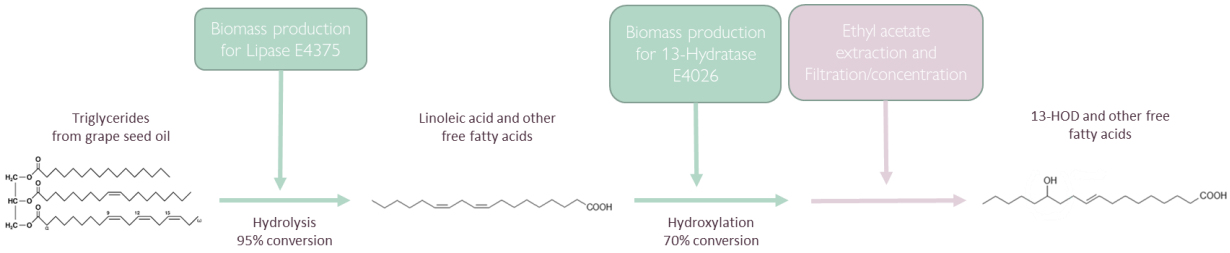

Figure 4 : Two-step biotransformation of grape seed oil to 13-HOD

The new active ingredient has been officially assigned the INCI name Hydroxylated Hydrolyzed Grape Seed Oil by the Personal Care Products Council (PCPC). Its biological efficacy has been confirmed through *in vitro* and *ex vivo* evaluation, with results consistent with those observed in the prototype. These results are detailed in Poster No. IFSCC2025-1634.

4. Discussion

Enzyme selection allowed us to identify E4014 10-hydratase and E4026 13-hydratase for the production of the four different prototypes. E4014 allowed us to obtain 10-HSA from oleic acid with more than 90% conversion without optimization. However 10-HOD from linoleic acid was obtained with only 20% conversion. Optimization of cofactors' recycling system and temperature allowed us to obtain 70% conversion for 10-HOD. For E4026, optimization of cofactors recycling system allowed us to obtain 13-HOD from linoleic acid with 70% conversion. Production of 10,13-DHOD was more challenging since 10,13-hydratase tested showed no activity on linoleic acid. We managed this reaction by combination of E4014 and E4026 and obtained 10,13-DHOD with more than 70% conversion.

Bbiological screening on PGE2, PGD2 and VEGF synthesis stimulation allowed us to identify 13-HOD as the most effective molecule. Actually, 13-HOD is the only prototype stimulating PGE2 synthesis without affectif PGD2 secretion, in comparison ricinoleic has no effect on both prostaglandins. Moreover, only 13-HOD stimulates the secretion of VEGF implicated in cells proliferation.Therefore, we focused on E4026 for the two-step biotransformation of grape seed oil.

Despite the triglycerides hydrolysis step of raw material grape seed oil, we were able to maintain a conversion rate of 70% from linoleic acid to 13-HOD. To achieve the target specifications of a minimum of 40% 13-HOD in the final ingredient, careful selection of the starting raw material grape seed oil is essential. As the process requires raw vegetal oil and native non optimized enzymes, the sourcing of high-quality grape seed oil rich in linoleic acid is critical to ensure the reproducibility and efficacy of the process.

Considering the use of biological products, the use of grape seed oil as a substrate combined with enzymatic biocatalysis, this process offers a sustainable and effective approach to developing hair growth-stimulating actives. Without intermediate purification step using organic solvents and highly selective natural catalysts, the enzymatic process is environmentally friendly and suitable for reproducible industrial production. The high concentration of 13-HOD achieved through this method demonstrates significant potential for commercial hair care products.

5. Conclusion

This study presents a novel and sustainable method for producing a hair growth-stimulating bioactive ingredient from grape seed oil. The enzymatic process ensures an environmentally friendly approach, and the resulting active ingredient shows promising hair growth-stimulating properties. Future work will focus on evaluating the *in vivo* long-term efficacy and safety of the active ingredient in hair care applications.

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