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List of Abbreviations and Acronyms	
FFA	Free fatty acids (FFAs)
PUFA	Polyunsaturated fatty acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic
OA	Oleic acid
LA	Linoleic acid

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1 Executive Summary

This deliverable presents the first of three planned deliverables under Task 2.4, focusing on the enzymatic synthesis of polyunsaturated fatty acid (PUFA) esters. The overarching aim of this work is to develop sustainable, mild, and selective biocatalytic processes that enable the stabilization and functional derivatization of PUFAs for applications in nutraceutical and cosmetic formulations. Enzymatic processes offer high selectivity, operate under mild conditions, and help preserve the structural integrity and bioactivity of sensitive fatty acids.

The work investigates enzymatic esterification of phytosterols with mono- and polyunsaturated fatty acids namely, oleic (OA; C18:1) and linoleic (LA; C18:2), as well as the conversion of microbial oils rich in eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6). Different biocatalytic routes—direct and indirect transesterification and hydrolysis-based esterification—were assessed to identify efficient and sustainable pathways for producing PUFA-enriched esters.

Overall, the results demonstrate that enzymatic transformation represents a viable strategy for generating high-value lipid derivatives. These investigations lay the groundwork for subsequent deliverables in Task 2.4, contributing to the broader ONE EARTH objective of advancing PUFA production and downstream transformation for next-generation nutraceutical and cosmetic products.

2 Context and aim of the study

Polyunsaturated fatty acids (PUFAs) are vital components of biological membranes and play multifunctional roles in maintaining health, particularly through their structural, metabolic, and signaling functions. Fatty acids containing two or more double bonds within their acyl chain are classified as PUFAs, and they are grouped into two major families based on the position of the first double bond: omega-3 (n-3, ω-3) and omega-6 (n-6, ω-6). Among these, the C18 PUFAs, namely linoleic acid (LA; C18:2 n-6) and α-linolenic acid (ALA; C18:3 n-3), serve as precursors for the synthesis of longer-chain derivatives such as arachidonic acid (AA; C20:4 n-6), eicosapentaenoic acid (EPA; C20:5 n-3), and docosahexaenoic acid (DHA; C22:6 n-3). These long-chain PUFAs are considered essential, as humans lack the desaturase enzymes necessary for their endogenous synthesis, necessitating dietary or topical supplementation.[1]

The skin's barrier function and immune balance rely heavily on adequate PUFA content. LA is the dominant omega-6 PUFA in the epidermis and an essential precursor for ceramide synthesis, which forms the intercellular lipid matrix critical for maintaining skin hydration and permeability. Deficiency in LA disrupts barrier integrity, leading to dryness and inflammation. On the other hand, omega-3 PUFAs such as EPA and DHA modulate inflammatory responses in the skin. These properties highlight the growing use of PUFAs in cosmetic formulations aimed at improving skin barrier function, alleviating dermatitis and acne, and promoting wound healing. Beyond dermatological benefits, PUFAs play critical roles in cardiovascular, neurological, and ocular health. Dietary intake or supplementation with ALA, EPA, and DHA supports membrane fluidity, signal transduction, and gene regulation, providing protective effects against inflammatory and metabolic disorders. [1,2]

Currently, the major commercial sources of PUFAs are fish oils, which are mostly produced from wild-caught fishes, rising concerns for the sustainability of fishing stocks and the depletion of marine resources. Subsequently, great effort is being made to find alternative strategies for PUFA production also considering the demand for vegan products. According to current projections, DHA availability may decline to the point where almost 90% population would face a shortage. Notably, there is a significant discrepancy between demand and supply, with the deficit anticipated to be more than 1 million tons. Researchers throughout the world are exploring alternative sources of PUFAs including microbes. [3] The microalgal content of EPA and DHA is far larger than that of plants. In addition, some species also contain substantial proportions of typical plant PUFAs such as C16:0 and C18:1, C18:2 and C18:3 thus attracting the attention of the biotechnological industry.[4]

PUFA are very sensitive to heat and oxidation, therefore strategies for their stabilization are needed. [5] Given their multifaceted bioactivities, PUFAs hold substantial promise for use in functional foods, dietary supplements, and cosmetic formulations. Their incorporation, stabilization, and bioavailability often depend on esterification and further formulation strategies, which can improve oxidative stability, sensory characteristics, and targeted delivery. Enzymatic catalysis, which proceeds efficiently under mild conditions, is attractive for the synthesis of esters of PUFA.[6] Therefore, ONE EARTH aims to focus on enzymatic esterification which is mild and highly selective technology with an aim to stabilize the PUFA and provide additional functionalities with wider application in nutraceuticals and cosmetics. The first target product during this phase of study is phytosterol esters of PUFA.

Phytosterols have a high melting point (>130 °C) and low solubility in both water and edible oils, which limits their application in food, medical, cosmetic, and other industries. To address this challenge, synthesizing phytosterol fatty acid esters is a common and effective solution. Esterified phytosterols exhibit better solubility in oils, lower melting points, and retain the beneficial properties of the original phytosterols. These esters can be easily incorporated into various fat-based foods and diets. Moreover, recent studies have shown that esterified phytosterols can lower blood total cholesterol and LDL cholesterol levels, like free phytosterols. [7]

The aim of this work was to investigate the enzymatic synthesis of phytosterol esters beginning with FFA of oleic and linoleic acids and later on extend the tools and methodologies to the esterification of microbial oils containing long chain PUFA. Eventually, the produced products will be tested for their potential application in cosmetics and nutraceuticals.

3 Materials and methods

First phase screening experiments were carried out using OA (C18:1) and LA (C18:2) as the FFA substrates for esterification with phytosterol. The aim was to establish baseline enzyme performance under solvent-free conditions. These screening trials were carried out on a 2 g scale, employing a set of six lipases at 1–5 wt% enzyme loading at FFA and phytosterol molar ratio of 8/1. Analysis was done by gas chromatography by measuring the decline of limiting substrate (phytosterol) in the reaction medium. Blank reactions without addition of enzymes were run in parallel.

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Once microbial oils were obtained, tests were conducted in a similar manner. First screening was done at 2 g scale, optimization at 10 g and reaction upscale with optimized enzyme at 300 g scale.

4 Results

4.1 First phase esterification trials with Oleic acid (OA; C18:1)

As shown in Table 1, lipase 3 and lipase 6, both immobilized formulations were the only active enzyme that showed conversion of phytosterol during esterification with OA. Based on this result, Lipase 6 and Lipase 3, were further investigated in section 4.1.1.

Table 1: Results of Initial Enzyme Screening Trials with Oleic Acid and Phytosterol

Lipase tested	Lipase form	Phytosterol conversion (%)
Lipase 1	immobilized	no conversion
Lipase 2	non-immobilized	no conversion
Lipase 3	immobilized	51%
Lipase 4	immobilized	no conversion
Lipase 5	non-immobilized	no conversion
Lipase 6	immobilized	95%

4.1.1 Further investigation of best performing lipases for FFA (OA; C18:1 and LA; C18:2) esterification with phytosterol

4.1.1.1 Effect of molar ratio

To optimize the substrate molar ratio, next tests were conducted at FFA and phytosterol molar ratio ranging from 2/1 to 8/1. A 2/1, 37% phytosterol conversion, was observed, which improved additionally at 4/1 and 8/1. The excess oleic acid improved phytosterol solubility, creating a more homogeneous reaction medium and enhancing enzyme–substrate contact. Moreover, the excess of fatty acid shifts the equilibrium toward ester formation. This combined effect of favorable equilibrium and better phase compatibility resulted in higher reaction rates and overall conversion efficiency in the solvent-free system. (Figure 1).

4.1.1.2 Effect of fatty acid unsaturation and phytosterol molar ratio

The results (Figure 2) show that at 2/1 molar ratio, phytosterol conversion during esterification with LA shows no significant conversion, while esterification with OA resulted in 37% conversion. When the molar ratio of FA to phytosterol is increased to 8/1, phytosterol conversions was also observed with LA (76%) albeit lower compared to OA (95%). These results indicate that monounsaturated fatty acids like oleic acid are more effective for phytosterol esterification than polyunsaturated ones such as linoleic acid, whose higher degree of unsaturation likely causes steric hindrance and oxidative instability, reducing its reactivity under the reaction conditions.

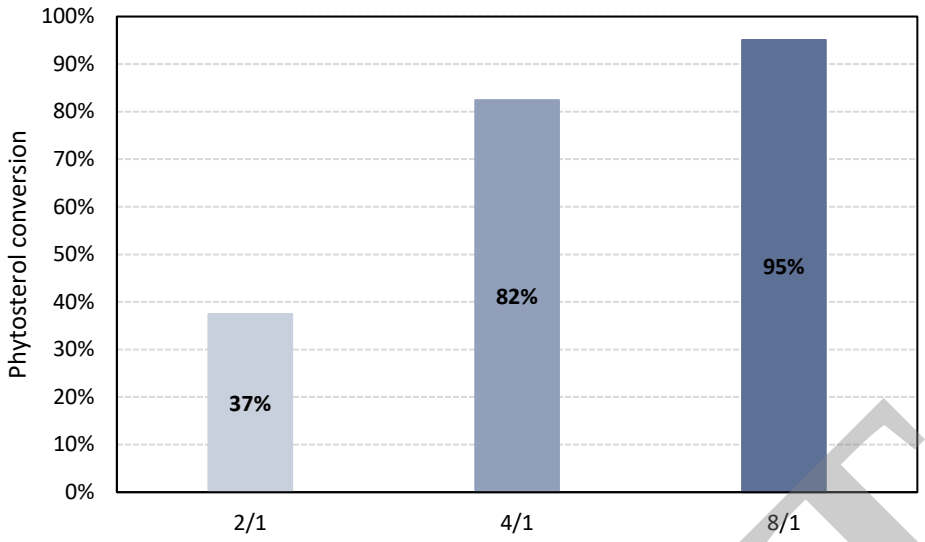

Figure 1: Effect of Oleic acid/phytosterol molar ratio on phytosterol conversion at 40°C using Lipase 6.

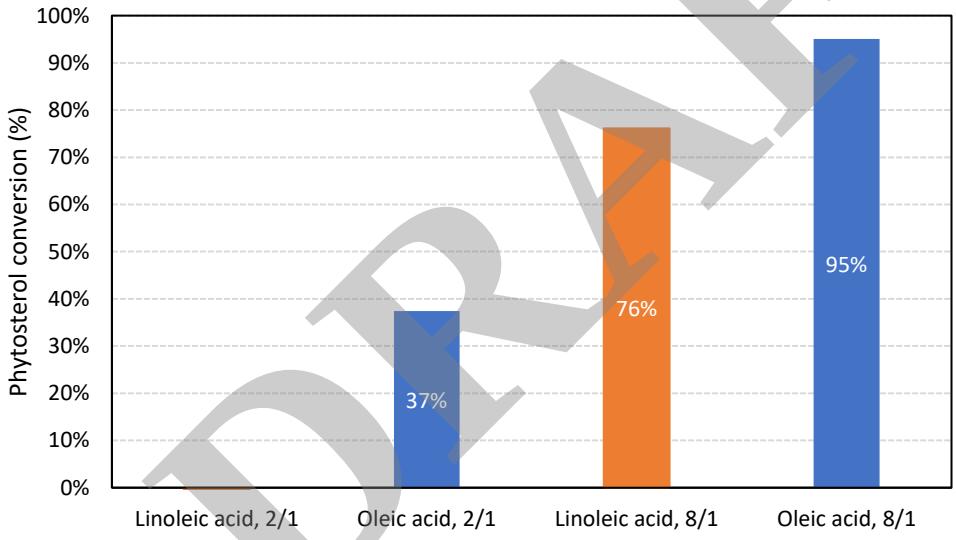

Figure 2: Effect of fatty acid unsaturation and molar ratios on phytosterol conversion at 40°C using Lipase 6

4.1.1.3 Effect of temperature

Effect of temperature was also investigated as it influences reagent solubility, enzyme–substrate collisions, and mass transfer efficiency. However, in this case (Figure 4), increase in temperature by 20°C did not improve the conversion. This suggests that Lipase 6 variant exhibit optimal activities over a broad temperature range and sufficient reagent solubilization was already achieved at the lower tested temperature. In addition, experiments were also performed with controlled water addition to assess its impact, since certain lipase-catalyzed reactions benefit from small amounts of water to maintain enzyme flexibility and facilitate substrate diffusion. However, in our study, no significant effect of added water was observed. It could be possible that enzyme already had a minimal essential

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hydration layer (bound water), and additional bulk water were unnecessary and perhaps even detrimental for condition at 60°C where it might have promoted hydrolysis over esterification.

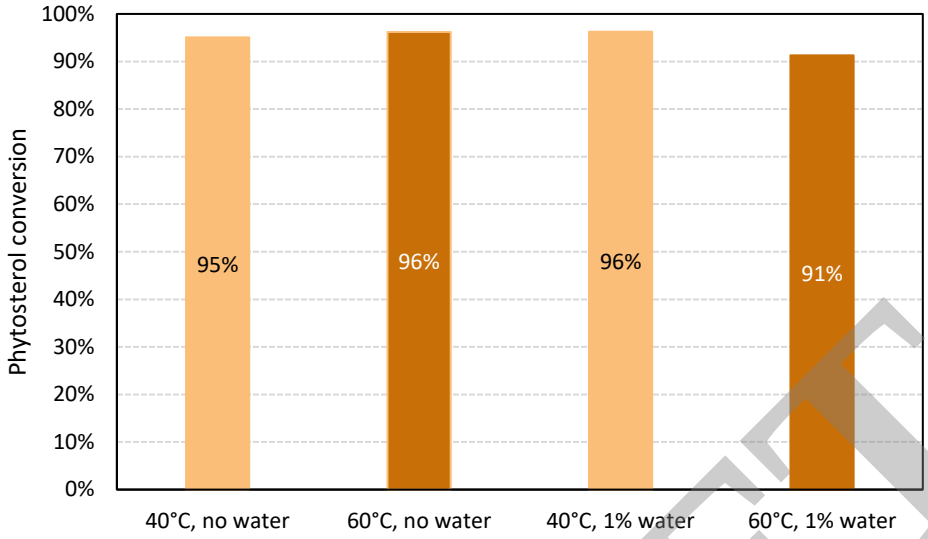

Figure 3: Enzymatic esterification of phytosterol and OA at 8/1 molar ratio.

Another candidate, Lipase 3, tested in first phase (Table 1) showed 51% phytosterol conversion, therefore it was also briefly tested to check if it shows better phytosterol conversion with LA (C18:2). However, the performance even at 8/1 phytosterol to LA molar ratio was lower (Figure 4), in comparison to Lipase 6 (Figure 2), therefore no further investigations were conducted for Lipase 3 for phytosterol esterification with LA.

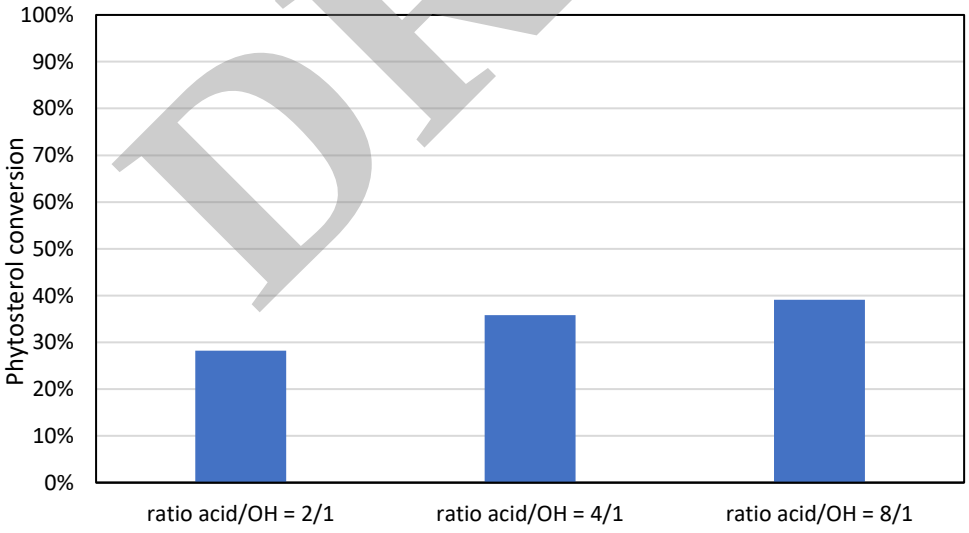

Figure 4: Effect molar ratios on phytosterol conversion during esterification with LA (C18:2) using Lipase 3

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4.2 Second phase trials for enzymatic transesterification of microbial oil with phytosterol

In the next steps, we used microbial oil rich in long-chain PUFAs, specifically eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), for further investigations. When using the oil as the substrate, three routes were considered:

- **Direct transesterification route:**
  In this approach, the microbial oil was directly subjected to transesterification with phytosterol, enabling the exchange of ester groups between the triglycerides in the oil and the phytosterols to form phytosterol esters and glycerol as a by-product.
- **Indirect transesterification route:**
  This route involved a two-step process. First, the microbial oil was converted into fatty acid alkyl ester. The produced alkyl ester was supplied as product for application testing in WP4. In addition, this alkyl ester will be transesterified with phytosterol to yield phytosterol esters.
- **Hydrolysis-based route:**
  In this pathway, the oil was initially hydrolyzed enzymatically to produce free fatty acids (FFAs) and glycerol. The resulting FFAs, rich in EPA and DHA, will be subjected to esterification forming the desired phytosterol esters.

4.2.1 Direct transesterification route:

Direct transesterification of microbial oil and phytosterol yielded conversions in the range of 8–12% across all tested lipases. Due to these relatively low conversion rates, this pathway was not pursued further. In the direct route, the enzyme must act on triglycerides, where the ester bonds are attached to the bulky glycerol backbone, making it harder for the enzyme to access the reaction sites for this oil.

4.2.2 Indirect transesterification via alkyl esters:

In this approach, the microbial oil was first converted into fatty acid alkyl ester before reacting with phytosterols. This two-step route was chosen to improve substrate accessibility and reaction efficiency. Alkyl esters are more reactive intermediate than triglycerides because the corresponding alcohol provides a better leaving group than glycerol, which facilitates the enzymatic transesterification step. Their simpler molecular structure and reduced steric hindrance also promote better enzyme–substrate interaction and higher catalytic efficiency. After initial screening, three enzymes were chosen for synthesizing alkyl esters of microbial oil. As shown in Figure 5, Lipase 1 resulted in complete conversion of microbial oil into alkyl ester. Both alcohol/oil ratios of 4.5/1 and 9/1 resulted in complete conversion of PUFAs to corresponding ester. For Lipase 7 and 5, manufacturer's recommended water additions, therefore tests were also conducted with 2% water, however the impact was negative for Lipase 7 and minimal for Lipase 5. Similar observations were made for higher alcohol/acid ratio for Lipase 7 and 5.

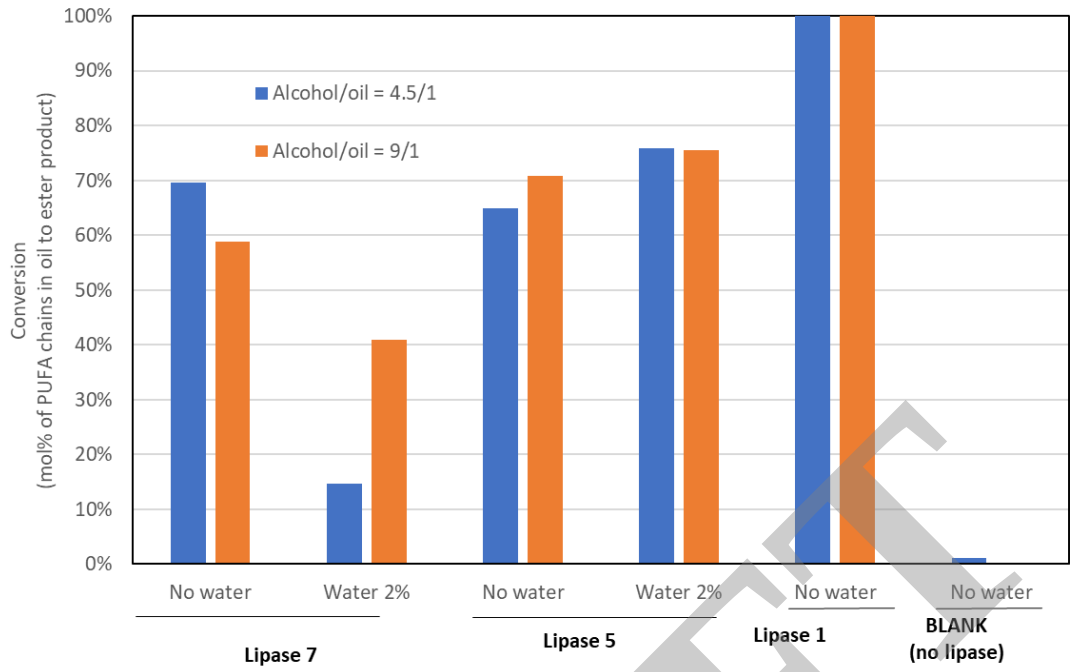

Figure 5: Conversion of PUFA into alkyl esters

Figure 6: EPA and DHA content in ester product

Moreover, for Lipase 1, the EPA (C20:5) and DHA (C22:6) content in the esters is comparable to that in the microbial oil (Figure 6), which is expected since complete conversion of PUFAs was observed. In contrast, for Lipase 7 and 5, where PUFAs were not fully converted to esters, the conversion initiates with EPA, followed by DHA. This indicates that the enzymes preferentially act on EPA, which has a shorter chain length and slightly lower degree of unsaturation, before processing the longer and more unsaturated DHA.

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Next tests involved subsequent optimization of reaction conditions for Lipase 1 (Figure 7). At 40°C, higher conversions obtained when alcohol was in excess. At 60°C, Stochiometric amount also led to complete transesterification of EPA and DHA chains. Coloration of samples was observed under air atmosphere which indicated onset of lipid oxidation; therefore, nitrogen atmosphere was selected for next tests. The optimized conditions also showed the EPA and DHA context like starting oil substrate (Figure 8).

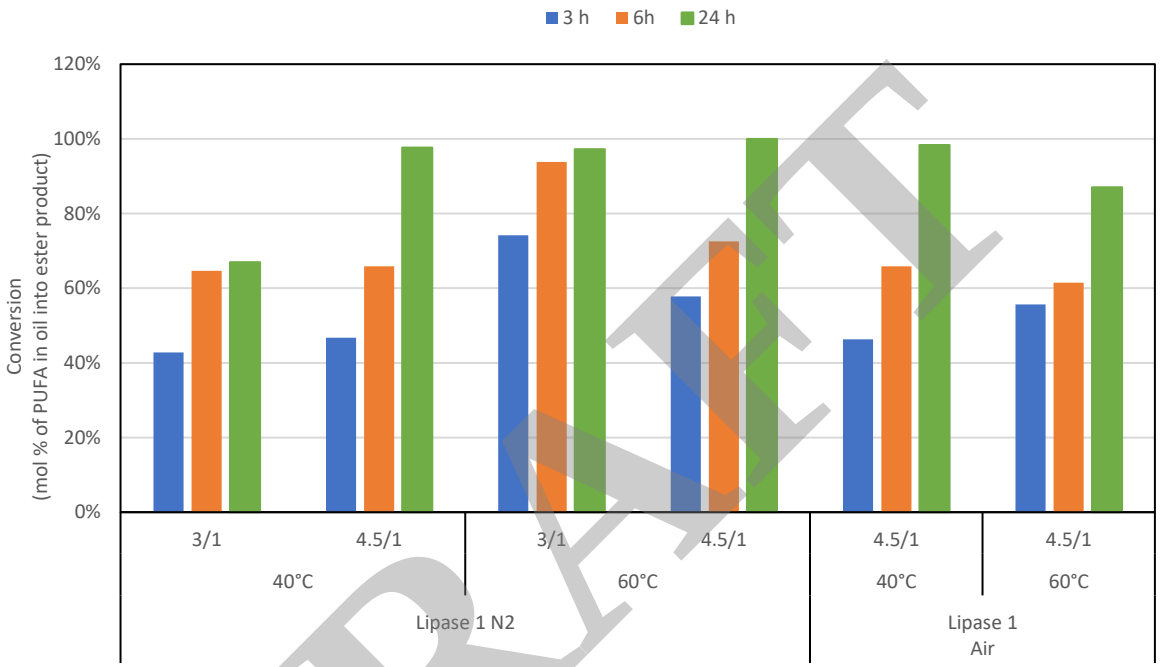

Figure 7: Optimization of alkyl ester production from microbial oil with Lipase 1.

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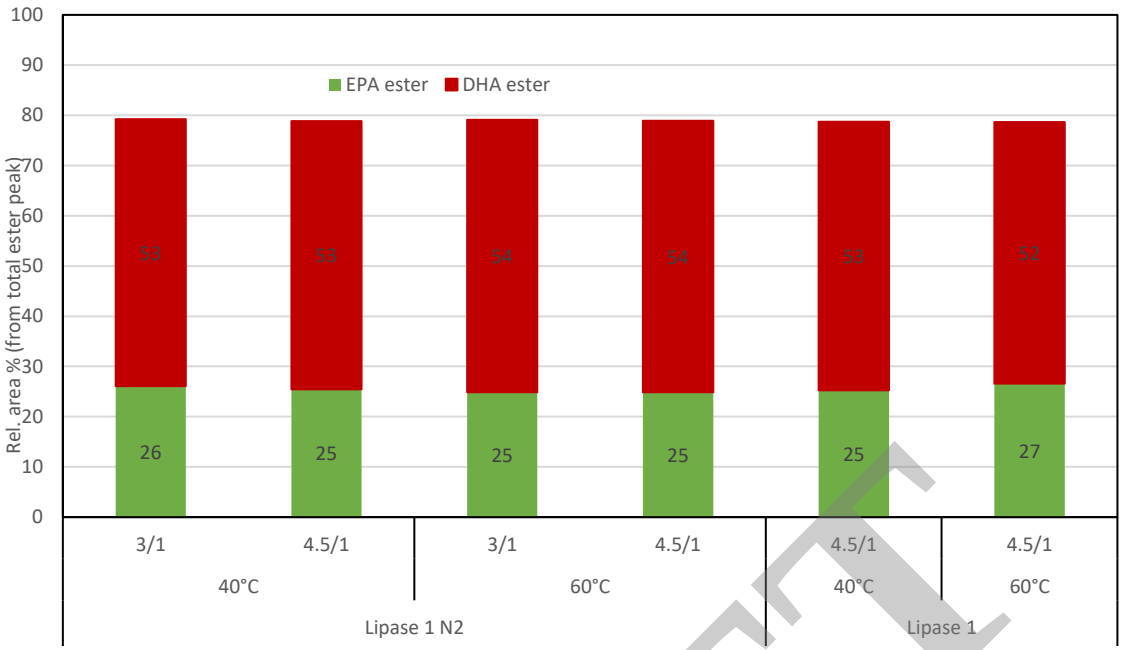

Figure 8: EPA and DHA ester proportion in alkyl ester product: reaction optimization with Lipase 1

The optimized conditions were later upscaled to 0.3 kg scale reactor and 100 g sample was prepared and sent for application testing in WP4 to **ANAVERIS** (Figure 9). In addition, these alkyl esters are currently being tested as substrate for phytosterol transesterification.

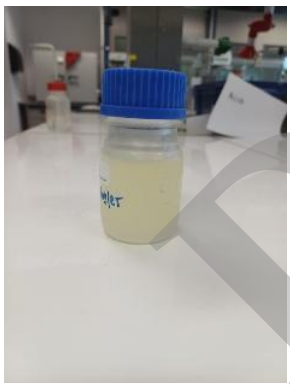

Figure 9: Alkyl ester of microbial oil: colorless/translucent sample sent for application testing

4.2.2.1 Esterification via hydrolysis route

In this approach, microbial oil was hydrolysed to release free fatty acids (FFAs) before esterification with phytosterols. This route simplifies the reaction system and enhances esterification efficiency. Hydrolysis breaks down triglycerides into FFAs and glycerol and making the FFAs more accessible and reactive substrates for sterol ester formation. As shown in the enzymatic hydrolysis results (Figure 10), Lipase 7 performed the best in addition to lipase 8 and 5, as opposed to alkyl ester synthesis where Lipase 1 was the most suitable catalyst.

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The tests with combination of lipases did not bring any extra benefit, therefore lipase 7 was selected for the next hydrolysis tests where FFAs of EPA and DHA will be generated for phytosterol synthesis. These tests have been conducted and currently in analysis. Moreover, as seen during alkyl ester formation, the composition of FFAs is also very similar to microbial oil indicating complete conversion of major PUFA chain to FFAs (Figure 11).

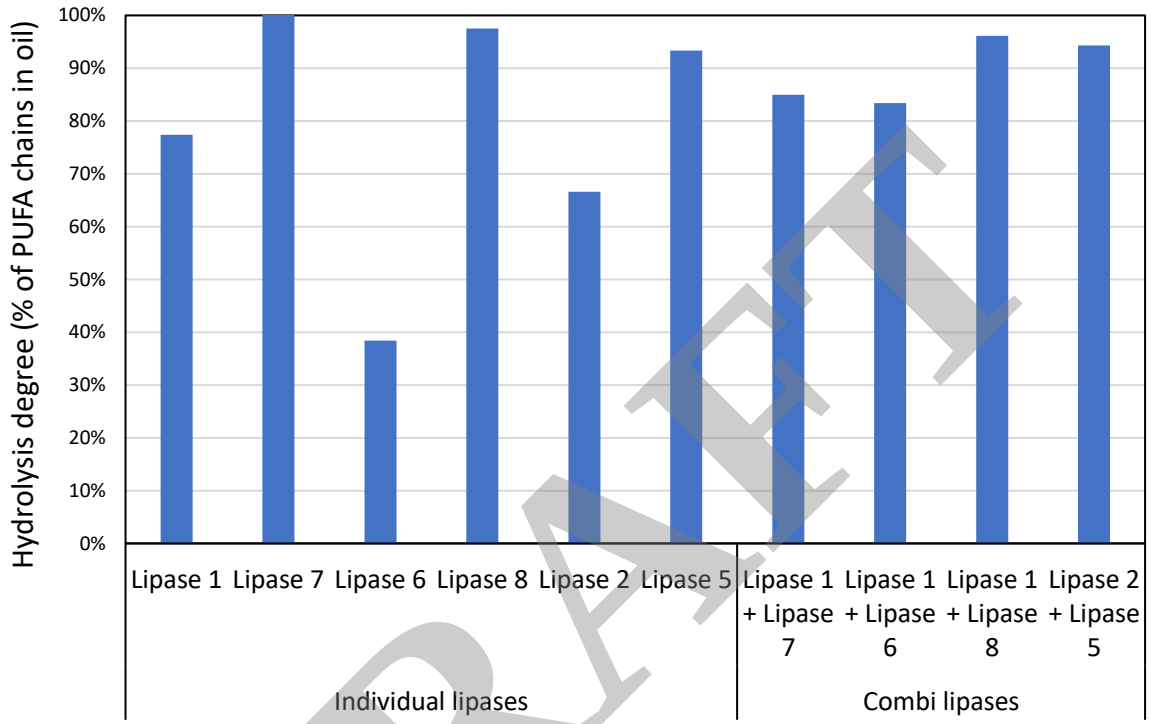

Figure 10: Hydrolysis of microalgae oil

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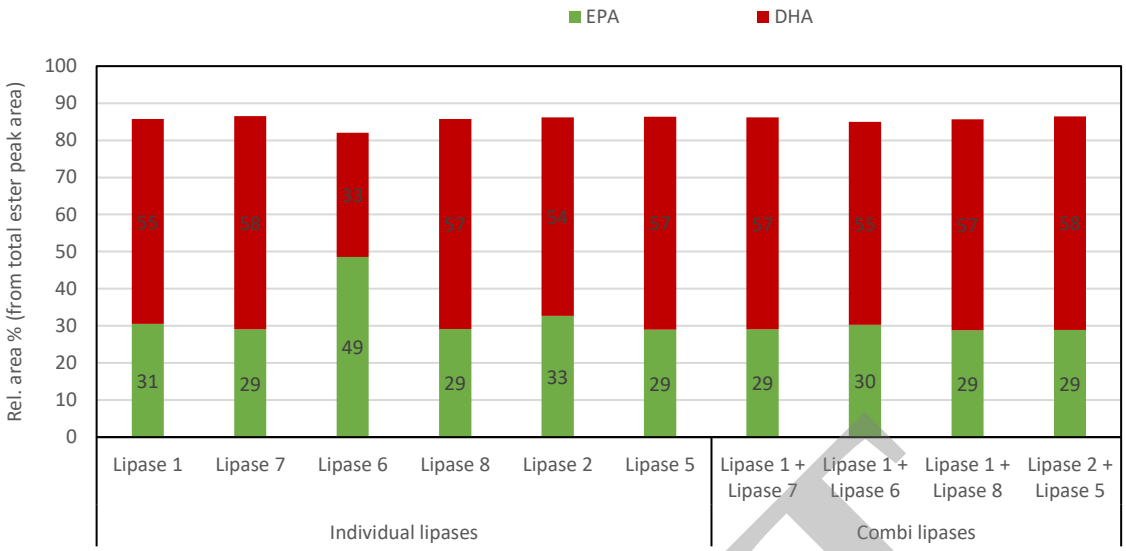

Figure 11: Composition of released FFAs after enzymatic hydrolysis of microbial oil

5 Conclusions

The first part of this deliverable investigated enzymatic synthesis of phytosterol esters from mono- and polyunsaturated fatty acids under solvent-free conditions. Immobilized Lipase 6 showed the highest activity for oleic acid (C18:1), achieving up to 95% conversion. Increasing the fatty acid-to-phytosterol ratio improved solubility and shifted the equilibrium towards ester formation. Monounsaturated fatty acids, such as oleic acid (C18:1), were more reactive than polyunsaturated linoleic acid (C18:2), likely due to reduced steric hindrance and better oxidative stability.

Using microbial oil rich in eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), the indirect transesterification route via alkyl esters proved most effective. Lipase 1 achieved complete conversion to alkyl esters, maintaining the EPA/DHA ratio of the parent oil. The enzyme showed preference for EPA—shorter and less unsaturated—before DHA. Optimized conditions were successfully scaled up to produce 100 g of alkyl ester product. The generated alkyl ester is also being used for transesterification with phytosterol

The hydrolysis route using Lipase 7 efficiently generated free fatty acids from microbial oil, providing a simpler system for subsequent esterification. Overall, the study demonstrates that enzymatic catalysis offers a sustainable and selective approach for producing PUFA-enriched phytosterol esters with potential applications in nutraceutical and cosmetic formulations.

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