proteus
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# GREEN ESTOLIDES
# ENZYMATIC SYNTHESIS OF FATTY ACIDS POLYESTERS & ESTOLIDES

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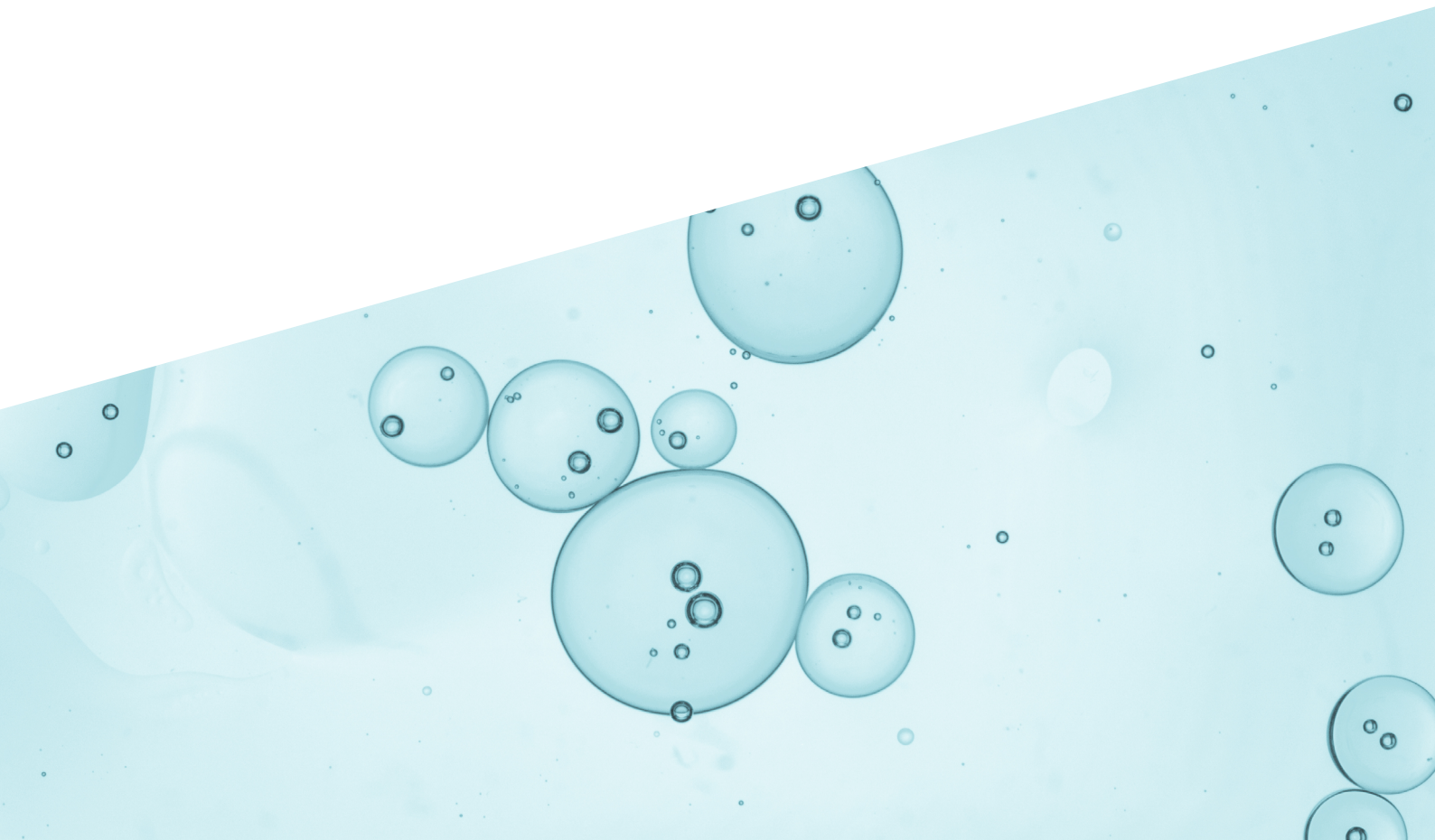


# 1-Introduction

Estolides are polyesters derived from fatty acids. They are biodegradable compounds which have industrial interest as lubricants, plasticizers, emulsifiers or moisturizers.
They find their application in the automotive, cosmetic and food industries. This wide field of applications is linked to their thermo-oxidative stability, their viscosity and their low melting point.

Estolides also have biological and texturizing properties and an adjustable hydrophilic / lipophilic balance. Estolides provide moisturizing enhancement to products, they act as a thermal barrier, they improve the elasticity of fibers, and they give a gloss effect. Cosmetic products can contain up to 20% of estolides.

Finally, capping the residual estolides functions, hydroxyl and carboxylic functions, can be performed to increase the stability of estolides and enhance the lubricant properties.

## 1.1 Background

Estolides synthesis is mainly performed by chemical processes from unsaturated fatty acids in the presence of a catalyst (sulfuric acid, perchloric acid, Montmorillonite). The estolides formed by these methods generally have a low purity and require lengthy and expensive purification steps.

As an illustration, US Patent No. 5,380,894 describes a chemical process for the formation of estolides from unsaturated fatty acids with Montmorillonite as catalyst and at 250 ° C. However no estolide composition is characterized.

Publication WO2013 / 009471 describes a chemical process for forming estolides from unsaturated fatty acids and perchloric acid as a catalyst. Although the description is intended to be very generic, the examples are limited to the use of oleic acid. It should be noted that these chemical processes do not allow the regiospecific targeting of the bond between the fatty acids of these estolides but lead to estolides containing mixtures of regioisomers. In addition, these chemical processes require elaborate purification processes for products intended for food, cosmetic or pharmaceutical applications.

An alternative process, leading to better regiospecific homogeneity consists of starting from hydroxylated fatty acids.

Indeed, patent publication WO2008 / 040864 describes a chemical process for forming estolides from hydroxylated fatty acids essentially using 12-hydroxystearic acid (12-HSA). The terminal acid function of the resulting estolid is then esterified in the presence of an alcohol.

Similarly, publication WO2011 / 037778 describes a process using 12-hydroxystearic acid. The terminal acid function of the estolid is then esterified in the presence of an alcohol and then the terminal alcohol function is protected with an aliphatic acid.

However, all these processes are carried out at high temperatures up to 210 ° C.

The enzymatic preparation of estolides has also been described. In 1983, during the hydrolysis of castor oil using a lipase from Geotrichium candidum, Okamura et al. have discovered the formation of estolides (ref 1) leading mainly to a mixture of monomers and dimers.

Within biocatalytic conditions, estolides are generally obtained from hydroxylated fatty acids. The prior art essentially shows examples with ricinoleic acid which is, together with 12-hydroxysteric acid, one of the two hydroxylated fatty acids available at industrial scale. The latter is not natural, it is produced by the hydrogenation of ricinoleic acid.

In 2009, Chan Woo Lee et al.2 oligomerized 12-HSA acid in a dean stark with Candida antarctica lipase B immobilized in benzene at 80 ° C, but the oligomers generated evolve into a cyclic dimer.

A single article describes the oligomerization of lesquerolic acid (a rare naturally hydroxylated fatty acid), in a two-phase medium and at room temperature, with lipases (non-thermostable) from Candida rugosa and Geotrichium candidum, Aspergillus niger and Penicillium cyclopium (ref 3) leading mainly to a mixture of monomers and dimers. In addition, the reaction times are very long, around 65 hours.

In 2012 (ref 4), Horchani et al. oligomerized ricinoleic acid with and without solvent using a thermostable biocatalyst (up to 70-80 °C). This biocatalyst is a lipase derived from Staphylococcus xylosus immobilized on calcium carbonate. However, the oligomerization of 12-hydroxystearic with a melting point of about 75-80 ° C has not been evaluated.

More recently, Martin-Arjol et al. (ref 5) claimed the oligomerization of 10-hydroxystearic acid at 80 °C without solvent with Novozym® 435 which corresponds to the lipase B of Candida antarctica immobilized on an acrylic resin. The conversion rate obtained is 70% in 168 hours under vacuum.

## 1.2 Objective

There is a need for the targeted applications to develop an efficient process to produce estolides under mild conditions in high yields and to avoid the formation of by-products without solvents.

To reach these expectations, Protéus by Seqens has developed an efficient enzymatic process from hydroxylated fatty acids with a lipase. According to prior art, no efficient enzyme has been identified to oligomerize hydroxylated fatty acids with a reaction temperature above 70°C.

Our first objective was therefore to identify one or several enzymes from the Protéus by Seqens's collection of our 350 ready to screen enzymes. The selected enzymes would need to perform oligomerization of the ricinoleic acid and the 12-hydroxystearic acid at its melting temperature (75°C-80°C) or beyond and without organic solvent.

# 2-Material and methods

## Biocatalysts preparation

All enzymes screened are expressed with E. coli. At the end of the culture phase, cells expressing the enzymes to screen are harvested and freeze-dried.

## Screening

The enzyme screening was performed on the ricinoleic acid without solvent and with 6% w/w of freeze-dried whole cells. The medium has been incubated at the optimal temperature for the enzymes under magnetic stirring. To assess the performance of the enzymes, the Acid Value (AV) and the Estolide Number (EN) were calculated from a representative sample of the reaction medium.

Acid Value (AV) – Estolide Number (EN):

The determination of the Acid Value (AV) has been performed with the titrator Mettler Toledo T50 equipped with the captor pH DG116-Solvent. The titrating agent is a solution of potassium hydroxide 0,1M in ethanol solution and the 3-methyl-2-butanone is used to dissolve the samples.

The Estolide Number (EN) corresponds to the average number of ester bonds of the product.

The AV and the EN calculations are done with the following formula :

$$AV = \frac{Veq\ X\ [KOH]X\ MW\ (KOH)}{m\ (sample)}$$

Units: Veq(m)L;[KOH](mol/L); MW(KOH](g/mol); m(sample) (g)

EN = (1000 X MW(KOH) X AV X MWsubstrate) -1

Units: MW(KOH) (g/mol); MWsubstrate (g/mol)

# 3-Results and Discussion

## 2.1 Screening of lipases

The screening has been performed with ricinoleic acid at the milliliter scale at the optimal temperature of the enzyme. After a 24-hour incubation, AV has been performed and EN has been calculated. Among the lipases and esterases of the collection of Protéus-by-Seqens, three candidates have allowed significant EN increase. The three selected lipases (E4032, E4375 and E4461) are from micro-organisms of the genus Streptomyces. The lipase E4461 is a thermostable mutant of the lipase E4375.

## 2.2 Comparison with lipase Novozym435®

The lipases identified have been evaluated with ricinoleic acid and the 12-hysroxystearic as substrates and their performance have been compared with the commercial lipase Novozym 435® described for its moderate ability to form estolides with fatty acids hydroxylated. The results of the oligomerization of ricinoleic acid (Figure 1) demonstrate that the biocatalyst Novozym 435® is not efficient enough to perform oligomerization of the ricinoleic acid. Furthermore, among the three lipases selected, the lipases E4375 and E4461 are the most efficient.

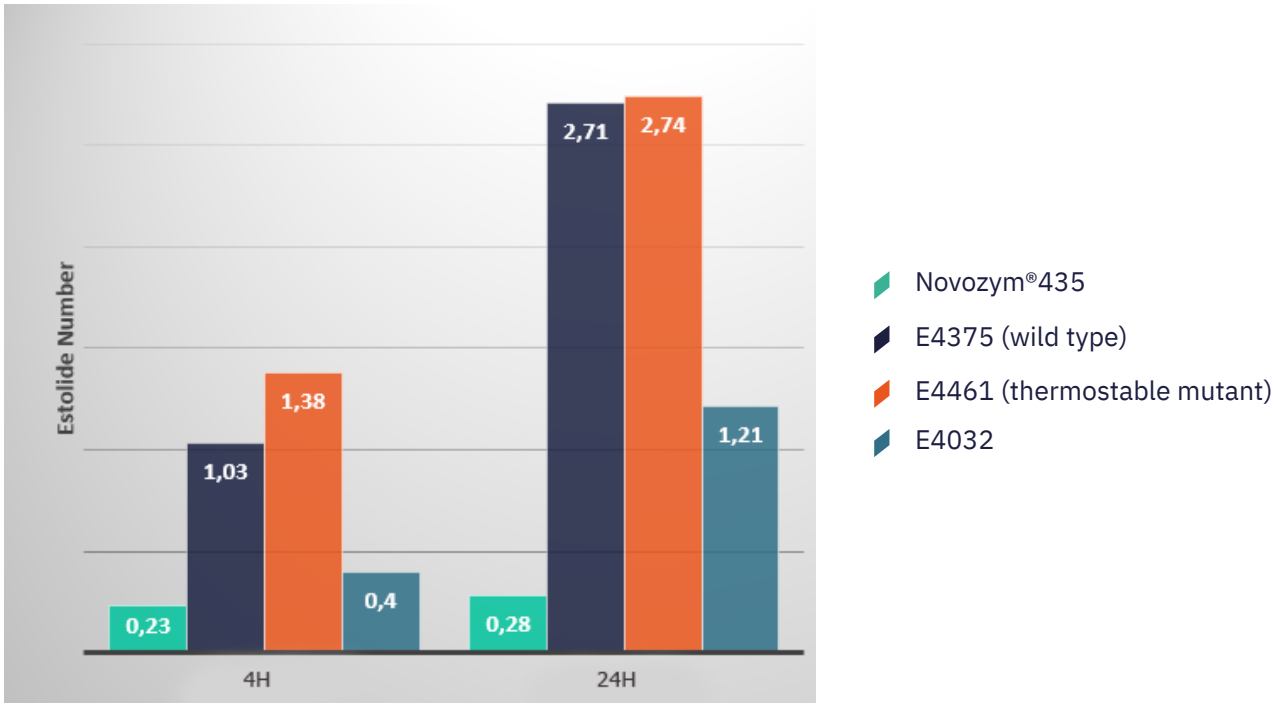



*Figure 1 : Comparison of the esterification results on the ricinoleic acid performed at 50°C during 24H with the 3 lipases selected and the Novozym 435®*

# 2.3 Optimization

The optimization of the experimental conditions was performed on the 12-hydroxstearic acid with the 2 promising lipases (E4375 and E4461) at two concentrations of the biomass in the reaction medium. A first assay has been conducted with closed flasks and open flasks to allow the water evaporation generated during the biotransformation .

The results (Figure 2 and Figure 4) show a significant improvement of the EN probably due to partial removal of water. At 24H, the EN in closed flasks at 80°C were respectively for the biocatalysts E4375 and E4461, 2.5 and 2.7 . In open flasks at 80°C, the EN were respectively 4.6 and 5.2.

Another test was performed with less biocatalyst (3.3%) and in open flasks. The results (Figure 3) demonstrate that it is not necessary to overload the reaction medium with biomass to obtain the desired EN: 3.3% w/w or less is enough.

A third test was run in the same conditions but in closed flasks with a nitrogen flow to strip the water generated. The results show similar EN at 24H with those in open flask.

Among all the runs, the biocatalyst E4461, a thermostable mutant of E4375 demonstrated a better performance than the E4375 at 4H or 24H.

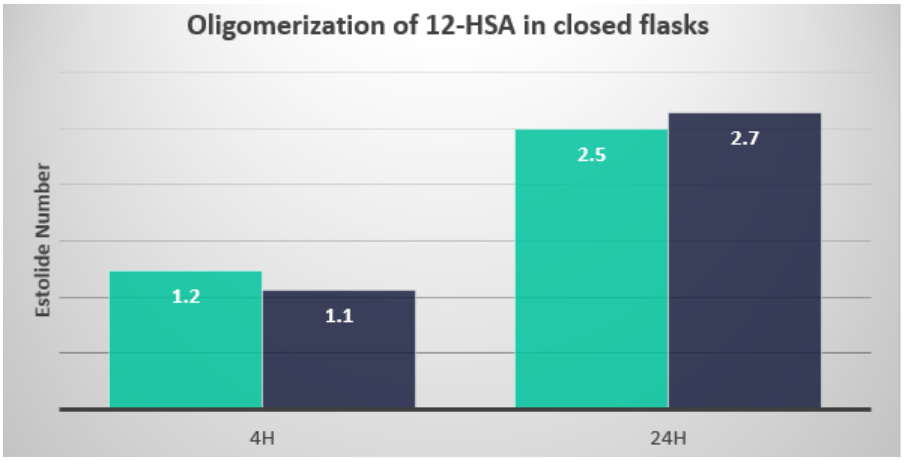



*Figure 2 : Results of EN number obtained for the oligomerization of the 12-HAS with E4375 and its thermostable mutant E4461 in closed flasks at 80°C with 5% of biocatalyst*

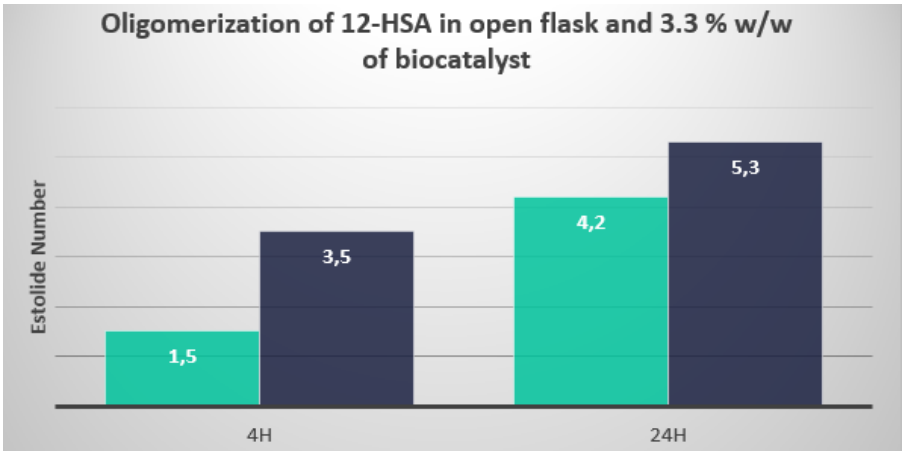



*Figure 3 : Results of EN obtained for the oligomerization of 12-HSA with E4375 and its thermostable mutant E4461 performed in open flasks at 80°C with 3.3% w/w of biocatalyst*

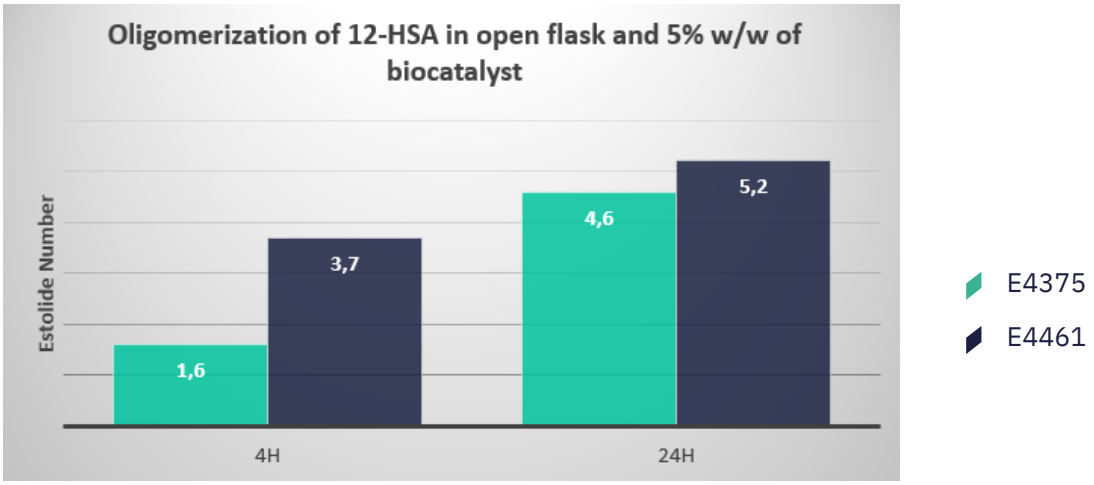



*Figure 4 : Results of EN obtained for the oligomerization of 12-HSA with E4375 and its thermostable mutant E4461 performed in open flask at 80°C with 5% of biocatalyst*

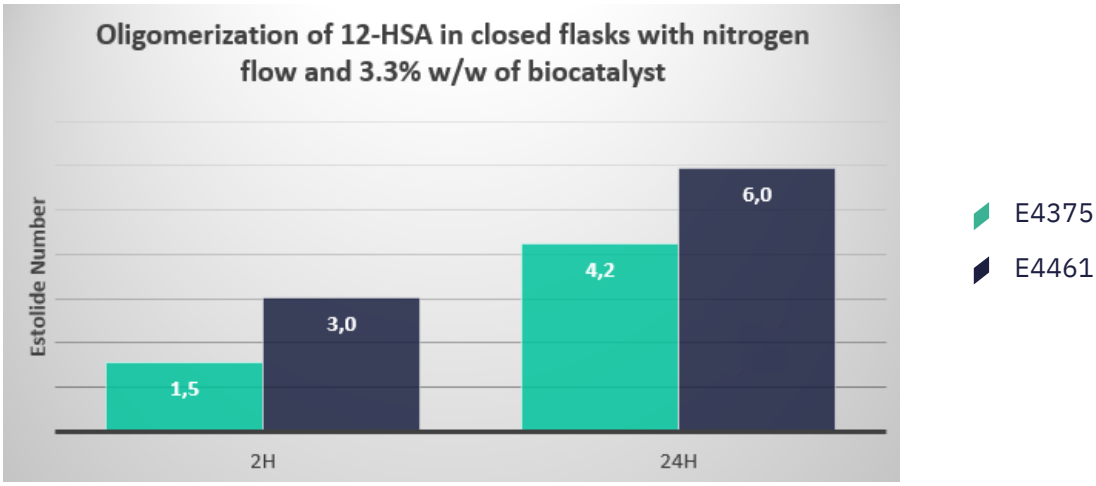



*Figure 5 : Results of EN obtained for the oligomerization of 12-HSA with E4375 and its thermostable mutant E4461 performed in closed flasks with a nitrogen flow at 80°C and 5% w/w of biocatalyst*

# 2.4 Capping 12-HSA

Lipase E4461 has also been studied for performing the capping of the 12-hydroxystearic acid with octanoic acid and 2-ethylhexanol.

Of course, to prevent the synthesis of the 2-ethylhexyl octanoate, the enzymatic reaction was conducted in two phases: first, esterification of the 12-hydroxyacid was performed with octanoic acid and then at the completion of the reaction, the 2-ethyl hexanol was added. To avoid the use of organic solvent the reaction was carried out at 80°C. To shift the reaction towards the esterification, water was stripped with a nitrogen flow.

Among all the runs, the biocatalyst E4461, a thermostable mutant of E4375 demonstrated a better performance than the E4375 at 4H or 24H.

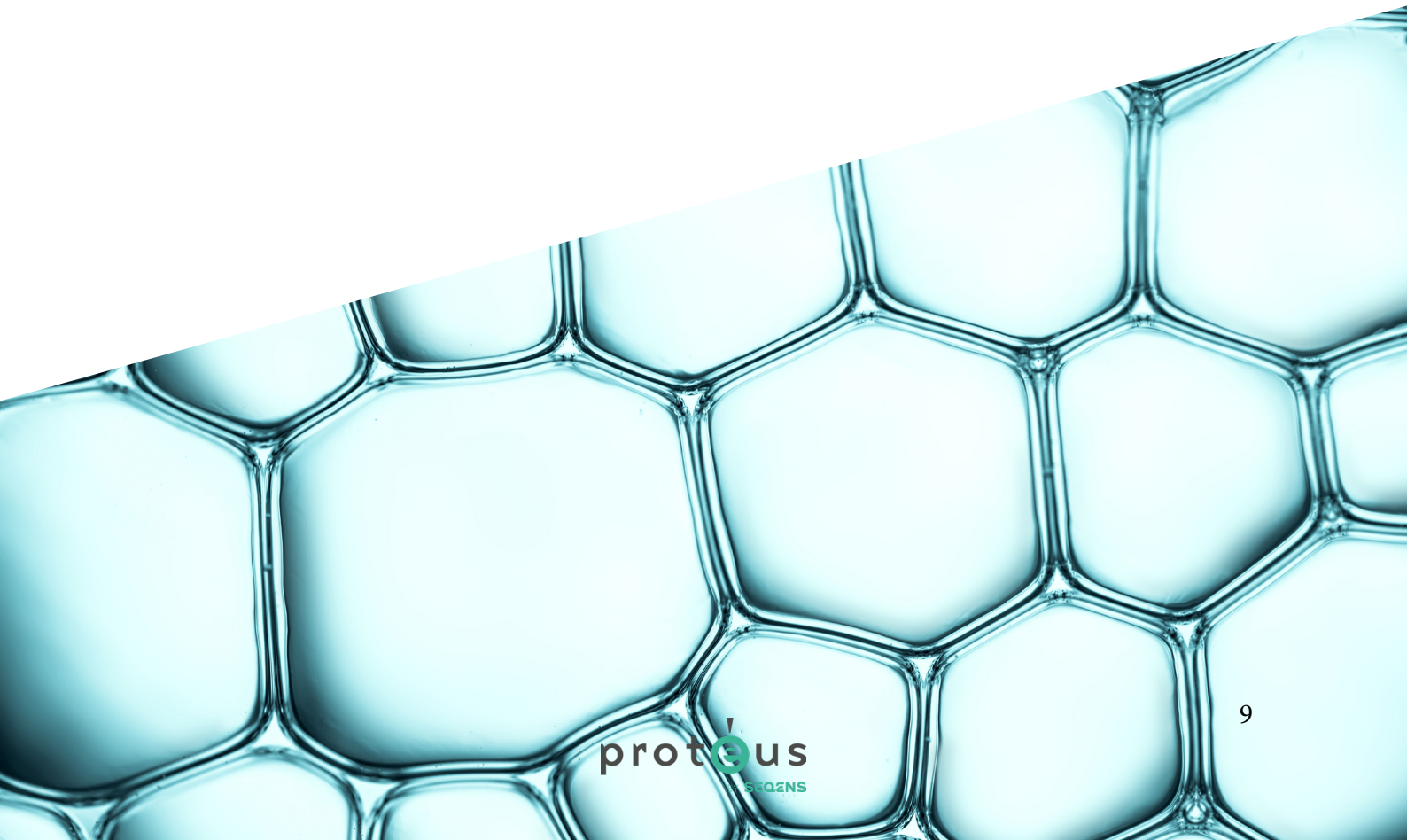
# 2.5 Scale-up

In order to assess the industrialization of the enzymatic process to form estolides, the first trials of 12-hydroxystearic acid oligomerization were carried out on a larger scale, at 1kg batch size. After the oligomerization step, capping conditions were implemented. ENs of about 2.7 were obtained with the E4461 biocatalyst and capping rates of residual alcohol and acid functions reached approximately 90%.

For the purpose of these tests, the E4461 biocatalyst therefore had to be produced on a larger scale, in 2-liter then 40-liter fermenters. To ensure robustness, process optimization was necessary in order to improve the production yields of the biocatalyst. Various parameters concerning the culture conditions were studied such as the composition and the pH of the culture medium, oxygenation, temperature and the duration of the culture. The best results in terms of growth, level of expression of protein of interest as well as esterification activity were obtained with commercial self-inducing media. However, lipase produced in minimal media allowed esterification of 12-hydroxy stearic acid. It was therefore important to pursue trials in order to find an alternative to the commercial auto-inducing medium which is too expensive and therefore cannot be transposed to an industrial scale.

To reach this objective, 3 sets of design of experiments (DoE) were implemented and they led to opitimzed conditions, especially on the composition of minimum culture medium leading to the production of the active lipase in the polymerization reaction within high productivity (>50g/L). A process book detailing the production conditions of the biocatalyst in fermentation conditions was then implemented for further scale-up.

Finally, productions up to multi-kg scale for the synthesis of estolides were successfully carried out. Depending on the nature of the estolides targeted (degree of oligomerization, nature of capping, etc.), the process can be adjusted, especially reaction time, to reach the required specifications.



# 4-Conclusions and Outlooks

For the enzymatic synthesis of estolides, Protéus by Seqens has identified several efficient lipases. They are able to oligomerize both ricinoleic acid and 12-hydroxy acid, the two most common and available hydroxylated fatty acids, performing without solvents and at high temperatures (up to 80 °C), considering their melting point temperature or beyond. These conditions enable the production of estolides with EN between 1 and 6 over a short reaction time. This work has been the subject of a patent application.

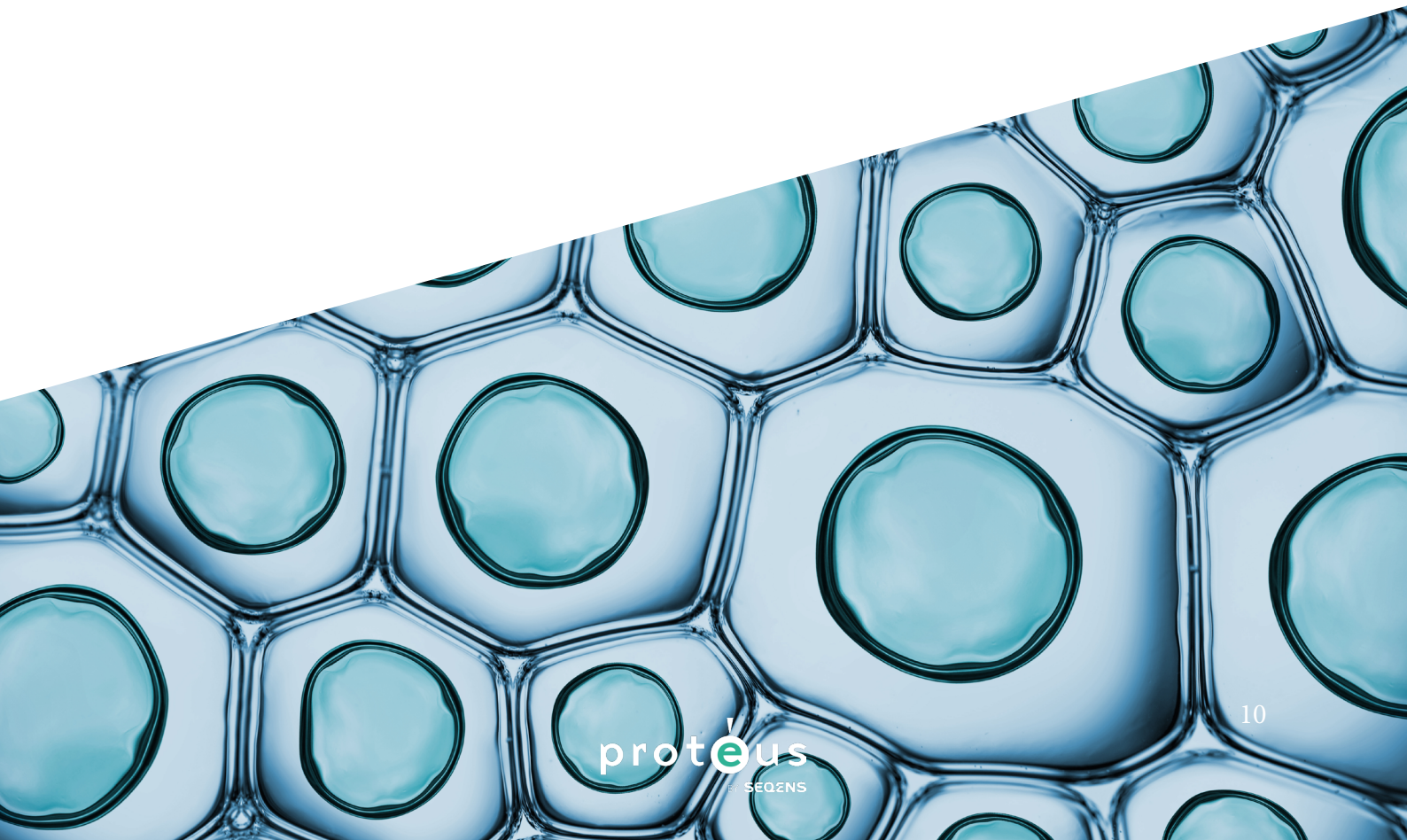
Moreover, the same enzymes are also able to cap hydroxy fatty acids or estolides with an organic acid and an alcohol in one pot.

For a robust and efficient industrial process, we have identified the key parameters, especially the need to respect the water content in the medium. Operational conditions were determined for such control.

These enzymes are available at Protéus for testing. We can also offer our expertise for any relevant problem solving you may require for implementing innovative and efficient process solutions.

Ricinoleic acid and the 12-hydroxystearic acid are among the few compounds that are available at industrial scale, within the hydroxy fatty acids derivatives.

To enlarge the panel of hydroxy fatty acids and consequently of the estolides, Protéus-by-Seqens has optimized hydratases allowing the synthesis of 10-hydroxystearic acid among others from unsaturated fatty acid as substrates.



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