Instituto Tecnológico y Estudios Superiores de Monterrey

Enhanced production of squalene in *Aurantiochytrium* sp. Yonez 5-1 by strain improvement with UV mutagenesis and treatment with terbinafine.

Biotechnological Process and Bioproduct Design

Group 504

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Executive Summary

The aim of the project is to enhance the production of squalene in *Aurantiochytrium sp*. Yonez 5-1 by strain improvement with UV mutagenesis and treatment with terbinafine. After identifying a niche in the squalene market to develop and offer a novel extraction methodology which can be not just scalable, but also environmentally sustainable, Vitality emerges. Vitality will provide cosmetic industries an oil-in-water nanoemulsion with a purity of 81% squalene.

The proposed bioprocess involves the fermentation of the microalgae, harvesting it, and extracting the lipids with a technology with lower energy consumption and high yields. The final product is achieved through a high-pressure homogenization where the nanoemulsification takes place, ensuring high purity and stability. Having complied with regulations and demonstrating that the project is financially viable, Vitality promises a high-quality, sustainable squalene production method.

1. Strategic Planning

1.1 Project Overview

Squalene is a natural triterpenoid hydrocarbon that acts as the biochemical precursor of sterols. It has multiple applications for human health and is currently used in cosmetic industries in its hydrogenated form (squalane) as a moisturizing agent and an emollient due to its anti-inflammatory, detoxifying, moisturizing and antioxidant properties (Yarkent & Oncel, 2022). It has also been used in the natural food supplement, medical, and pharmaceutical sectors due to its effectiveness in inhibiting tumor proliferation and improving the immune system function (Saengwong *et al*., 2018).

Traditionally, squalene has been extracted from shark liver oil. However, this source presents several problems, such as the impact on shark populations due to overfishing, ecosystem damage, the presence of contaminants in the oil, and unpleasant odors and tastes (Fracchia-Durán *et al*., 2023). As a result, restrictions have been imposed on marine animal poaching under the Marine Mammal Protection Act Policies (Markets and Markets, 2023). Vegetable oils have been the second commercial source of squalene production, however, their production yields are low because of their low squalene content (Popa *et al*., 2015).

Therefore, there is a need for novel sources that can provide adequate supplies of squalene at a low cost. It has been shown that microalgae can produce squalene. Some thraustochytrids, particularly species belonging to the genus *Aurantiochytrium*, produce squalene in quantities of more than 30% of the cell dry weight (CDW).

Our company, Vitality, emerges as an alternative for cosmetic and personal care product manufacturers in Mexico who are shifting towards cruelty-free and vegan ingredients, as well as those demanding hydration and anti-aging formulations. Vitality will provide them with an oil-in water nanoemulsion containing squalene at 81% purity as a valuable ingredient for cosmetics formulations. For this purpose, the project aims to enhance and optimize the production of squalene in *Aurantiochytrium sp.* Yonez-1 through UV mutagenesis and terbinafine treatment, along with the development of an oil-in-water (O/W) nanoemulsion to increase the final product´s stability, bioavailability and skin delivery. The feasibility of the project will be assessed through technical, economic and regulatory evaluations.

1.2 Project Justification (Innovation and Impact)

There's a need to identify and create sustainable sources of squalene that can be used commercially. Among the microorganisms studied for squalene production, microalgae stand out due to their high productive concentration. However, there are several limitations such as the cost of cultivation, cell harvesting, and compound extraction. Therefore, improving the performance of cell factories to increase yield of high value products is a promising approach to boost production efficiency. It has been reported that enhancing a strain can increase yields and productivity by up to 50% compared unmodified microalgae (Potijun *et al*., 2021). Strain improvement for higher product yield and tolerance to harsh environments is an attractive method for obtaining squalene from microalgae, as this process is more sustainable, less expensive, and yields higher amounts compared to the current methods on the market.

It has been applied to microalgae and other oleaginous microorganisms to produce resistant strains with improved lipid production. Additionally, the expression of important genes involved in fatty acid biosynthesis has been reported after UV mutation (Liu *et al*., 2015). Following UV mutation, *Aurantiochytrium sp.* Yonez 5-1 will be treated with terbinafine, a highly lipophilic fungicide that has been used to boost squalene levels in *Aurantiochytrium* by adding it to the culture medium.

The antimycotic inhibits the activity of squalene monooxygenase, a key enzyme in sterol biosynthesis that catalyzes the oxidation of squalene to 2,3 oxidosqualene (Ono, 2002). By inhibiting substrate binding to the active site of the enzyme, it may prevent further degradation of squalene, leading to increased

accumulation of squalene in the cell (Fan *et al*., 2009). The methodology followed for UV-C mutagenesis and terbinafine treatment is described in appendix 1.

As an added value to the final product, an O/W nanoemulsion will be developed. Nanoemulsions are a more suitable delivery system for the transport of lipophilic compounds, as they support the skin penetration of active ingredients and thus increase their concentration in the skin. This improvement plays an important role in cosmetics product formulations (Chellapa *et al*., 2016).

1.3 Product Description

At Vitality, our unique and exclusive product is AquaVit*,* an O/W nanoemulsion rich in squalene (81% purity) and antioxidants. The attributes offered by the nanoemulsion enhance the absorption and permeability of squalene and antioxidants in the skin. *AquaVit* is versatile and can be added into various cosmetic formulations, including moisturizers, facial creams, anti-aging creams, shower oils, lotions, ointments, lipsticks, eye makeup, and sunburn treatments. It can even be used as a single ingredient. The product will be packaged in 2.5 gallons polyethylene vacuum sealed buckets (shown in appendix 2), each properly labeled as shown in Figure 1. Aquavit's technical data sheet is provided in appendix 3.

Figure 1. Final Product label.

1.4 Market and Plant Capacity

Approximately 70-80% of the total produced squalene is consumed by the cosmetics industry (Grand View Research, 2022). Therefore, our market is aimed at cosmetics and personal care industry, which dominated the squalene market in terms of both value and volume in 2023 (Markets and Markets, 2023). In Latin America, the global cosmetics and skin care industry market represents 8% of the global market,

with a value of $7.24 billion (Statista, 2024). Mexico represents 2.66% of the global cosmetic market, making it a significant potential market. A closer examination of Mexico´s cosmetics market reveals that skin care products are the most popular, accounting for 42% (Statista, 2022).

In recent years, many companies have begun using squalene as an ingredient in their skin care products (Yarkent & Oncel, 2022). Since Mexico´s squalene market remains relatively untapped, there is an opportunity to target new customer niches. Currently, Mexico is home to approximately 77 companies who manufacture cosmetics and personal care products, including industry giants like L'Oréal, AVON, and Natura, all of which represent potential markets for our product.

The anticipated covered demand will be 8,250.00 kg annually, representing 11% of the cosmetic market in Mexico, a volume that aims to meet the demand generated by Uniliver Group, a leading company in cosmetics and personal care in Mexico, with a market share of 10.8% (ICEX, 2022). Simultaneously, the plant is designed to produce 9,016.50 kg annually, with the potential to increase the covered demand by 1% of the anticipated demand.

1.5 Plant Location

An analysis was conducted to identify a suitable site for our industrial plant. Four different states were evaluated: Querétaro, Jalisco, Monterrey, and Estado de México. The most important considerations for selecting the plant location were: 1) Nearness to raw materials, 2) Nearness to markets, 3) Climate conditions, 4) Availability of water, 5) Availability of workforce, 6) Rent costs, and 7) Protection against natural events.

A decision matrix with weighted criteria was developed to systematically evaluate these factors for each location. The results of this matrix, which are detailed in appendix 4, indicate that Jalisco is the most suitable state for our plant location. Consequently, Vitality will be placed in Metropolitan Circuit Industrial Park in Tlajomulco, Jalisco.

2. Technology Planning

2.1 Available Technology

The development of an effective and energetically efficient lipid extraction process from microalgal cells is critical for downstream process and production costs. To identify promising and effective extraction methods for obtaining squalene from microalgae, a decision matrix was made (shown in appendix 5). Supercritical CO_2 extraction has been widely considered by many researchers as the ideal

fluid for extraction and separation processes due to its non-toxic, non-inflammable, and non-polluting nature (Lee *et al.*, 2012). However, it yields a purity of over 95% for squalene (Halim *et al.,* 2021), which exceeds the purity required for the cosmetic industry (70%). Other technologies offer the required purity with lower investment and energy costs.

Organic solvent extraction, while low-cost and high yield, poses environmental and health risks due to solvent usage (Correa *et al.*, 2020), which contradicts the sustainable methodology desired for squalene extraction. Therefore, the most suitable technology is extraction using a High Shear Mixer (HSM) with ethanol as the solvent. This technology eliminates the energy-intensive drying step, reducing both energy consumption and the amount of solvent required for lipid extraction. HSM can achieve cell disruption and lipid extraction simultaneously, yielding high lipid yields at an industrial scale (Kwak *et al.*, 2019).

2.2 Flowchart and process description

Vitality´s biotechnological process begins with an UV-C mutagenesis for the improvement of the microalgae strain in an off-site laboratory. Subsequently, 7-unit operations will be done in the proposed industrial plant. Microalgae cultivation begins in a seed bioreactor to reduce the lag phase, as the microorganism first adapts to the controlled condition in a smaller volume. By employing this technique cellular proliferation is accelerated, ensuring that upon transfer to the primary bioreactor, they enter their exponential growth phase (Yan, *et a*l., 2016). Therefore, the main fermentation is continued with the addition of the seed bioreactor output along with new culture medium with optimum glucose concentration, seawater and nitrogen sources for proper growth (Nakazawa, *et a*l., 2012). After fermentation, a disk-stack centrifuge is used to harvest the produced biomass.

The most prevalent centrifugal device identified as effectively useful to harvest algal biomass is the disc-stack centrifuge (Najjar, *et al.*, 2020).

Lipid extraction in a high shear mixer with ethanol as solvent was the technology of choice as previously mentioned, due to the high lipid extraction yields and low energy consumption compared to other methods (Kwak, *et al.*, 2019). For the removal of cell debris after cell disruption, another disc-stack centrifuge will be used. Moreover, ethanol was recovered by using a flash distillation. This technique is useful for separating components of a mixture, such as organic solvents and oils with different boiling points (Perry, 2008). Since the oil still carries a large amount of water thar couldn't be removed in the previous centrifugations, a tubular centrifuge will be used to perform an efficient and continuous separation of both immiscible liquids (Agustyn, 2024). Finally, as an added value to our final product with

squalene at 81% purity, a high-pressure homogenization will be performed to obtain an oil-in-water (O/W) nanoemulsion with lecithin as a surfactant. Therefore, the final product obtained from the homogenizer will be characterized by having a small particle size, facilitating greater absorption into the skin and ability to reduce the water loss from the skin (Chellapa, *et al.*, 2016).

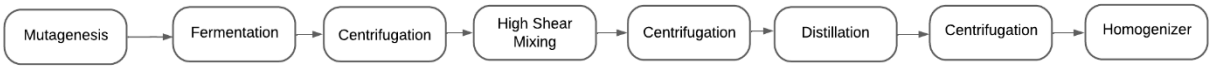


Figure 2. Process Flowchart

2.3 Mass Balance

The downstream processing begins with 746.36 g of *Aurantiochytrium sp.* Yonez 5-1 inoculum in the seed bioreactor. For both bioreactors, a stoichiometric balance was performed to determine the amount of CO_2 and H_2O generated. The biomass generation and substrate consumption were derived from microbial kinetics (see appendix 6). The empirical biomass formula was obtained from the elemental composition of *Aurantiochytrium sp.* reported by Kwaw, *et al. (*2019), and was associated with aerobic growth without product generation equation for microalgae. Mass balance for each unit operation and general mass balance of the process to produce an O/W squalene nanoemulsion are reported in appendix 7.

Considering the total yield of the process (pl) to produce an O/W nanoemulsion with a final squalene purity of 81%, the amount of product per batch would be 1,009.58 kg with 245.97 kg of squalene each one, as shown in Table 1.

Table 1. Yield recovery and Purity of squalene in each unit operation.

Unit Operation	Yield	Squalene Recovery (Kg)	Squalene Purity (%)
Fermentation	N/A	305.02 (in wet biomass)	N/A
Disc-Stack Centrifuge	97%	296.13 (in wet biomass)	N/A
High Shear Mixer	96%	287.51	12%
Disc-Stack Centrifuge	97%	278.88	13%
Distillation	99.50%	277.49	37.9%
Tubular Centrifuge	98%	273.30	81.1%

High Pressure Homogenizer	90%	245.97	81.1%
Total	79.27%	245.97	81.1%

2.4 Energy Balance

The energy consumption per batch was obtained with the energy balance for each unit operation (major and minor equipment). Table 2 reports the required energy for every equipment, as well as the total energetic consumption per batch and per year, considering for the latter the total annual working hours to satisfy the demand. The calculations can be observed in Appendix 8.

Table 2. General energy balance per batch and per year to produce an O/W squalene nanoemulsion.

Equipment	Energy (kW)	Time (hours/batch)	Consumption (kWh/batch)	Consumption (kWh/year)
Seed Airlift Bioreactor	2.60	147.25	646.17	23262.12
Airlift Bioreactor	21.93	223.33	4897.50	176310
Disc Stack Centrifuge (DS-101)	15.63	4	62.50	2250
High Shear Mixer	18.38	3.16	58.07	2090.52
High Shear Mixer Heating	17.67	3.16	55.84	2010.24
Disc Stack Centrifuge (DS-102)	15.63	1.2	18.75	675
Distillation	199.05	2.11	420.00	15120
Tubular Centrifuge	3.85	4	15.42	555.12
Emulsifying High Pressure Homogenizer	40	14	560.00	20160
Packaging	0.98	0.83	0.81	29.16
Total Mixing Tanks	1654.11	Various	4972.13	178996.68
Total Pumps	13.73	Various	15.25	549
Total Electric Heat Exchangers	1559.75	Various	642.83	23141.88
Total Boiler Heat Exchangers	2261.38	Various	2211.24	79604.64
Total Consumption			tuber	524,754.36

3. Engineering Design

3.1 Process Diagram

The process diagram to produce an O/W squalene nanoemulsion was performed by using Super Pro Designer software as shown in Fig. 3. The process begins with 3 streams (S-004, S-003, S-006) to the Seed Airlift Bioreactor (AFR-101). The stream S-004 contains the inoculum at a concentration of 2g/L; S-005 contains the culture medium previously sterilized in V-101, its composition is shown in appendix 9; S-006 contains an air flow rate of 0.0079 m^3/s, which is fed by a gas compressor (G-101). Output S-007 represents the CO_2 flow produced by the aerobic growth of the microorganism. After 146 hours of fermentation at 28°C with 2 feeding cycles (S-005) of glucose at a concentration of 250 g/L, the entire volume is transported to the main Airlift Bioreactor (AFR-102) through a peristaltic pump (B-3). Additional to the S-009 stream, new pre-sterilized culture medium (S-012) and an air flow rate of 0.07 m^3/s (S-013) enter AFR-102. Fermentation time lasts 223 hours at 28°C with 9 feeding cycles (S-014) of glucose at 250 g/L. Afterwards, the obtained volume is pumped (B-6) to a disc-stack centrifuge (DS-101) for biomass harvesting at 9,000 rpm for 4 hours. Since the centrifuge operates continuously, there will be a storage tank (V-103) for the collected biomass.

A stream of ethanol (S-020) flows into V-103 for the upcoming lipidic extraction. Through a peristaltic pump (B-8), the flow is conducted to four different high shear mixers (HSM-101, HSM-102, HSM-103, HSM-104).

Each HSM will operate for 2 cycles at 1800 rpm, for 6 hours and 30 minutes. Then, the 4 streams (S-021A, S-021B, S-021C, S-021d) are pumped through B-9 and enter the disc-stack (DS-102), which operates continuously at 9,000 rpm for 4 hours. Generated cell debris by previous cell disruption is discarded (S-023) and the supernatant is recovered (S-024).

Afterward, S-024 passes through a heat exchanger, reaching 85°C before entering the drum distillation tank (C-101). The stream is fed into C-101, where the vapor phase, a mixture of ethanol and a fraction of water, exits the system through S-025, while the liquid phase remains at the bottom of the drum and is subsequently cooled by a water chiller through S-026. Stream-026 enters the tubular centrifuge (BC-101) to separate the remaining immiscible phases composed of water and oil. BC-101 operates continuously at 5,200 rpm for 4 hours. The fraction containing the water (S-027) is recovered and fed to the mixing tank (V-105) where it is mixed with the S-019, where the surfactant enters, at 100 rpm and 50°C. This step is prior to the formation of the O/W nanoemulsion.

Simultaneously, the fraction with the oil will be taken to a storage tank. Once the mixture is obtained, both streams (S-28B and S-030) will be pumped to the High-Pressure Homogenizer for the formation of the final product. In each cycle, the mixture will be stored in V-106 and V-107. Once the cycle is completed, the obtained emulsion will be recirculated through a peristaltic pump (B-14) to accomplish the remaining cycles. The resulting O/W nanoemulsion leaves through S-034 and is collected for packaging.

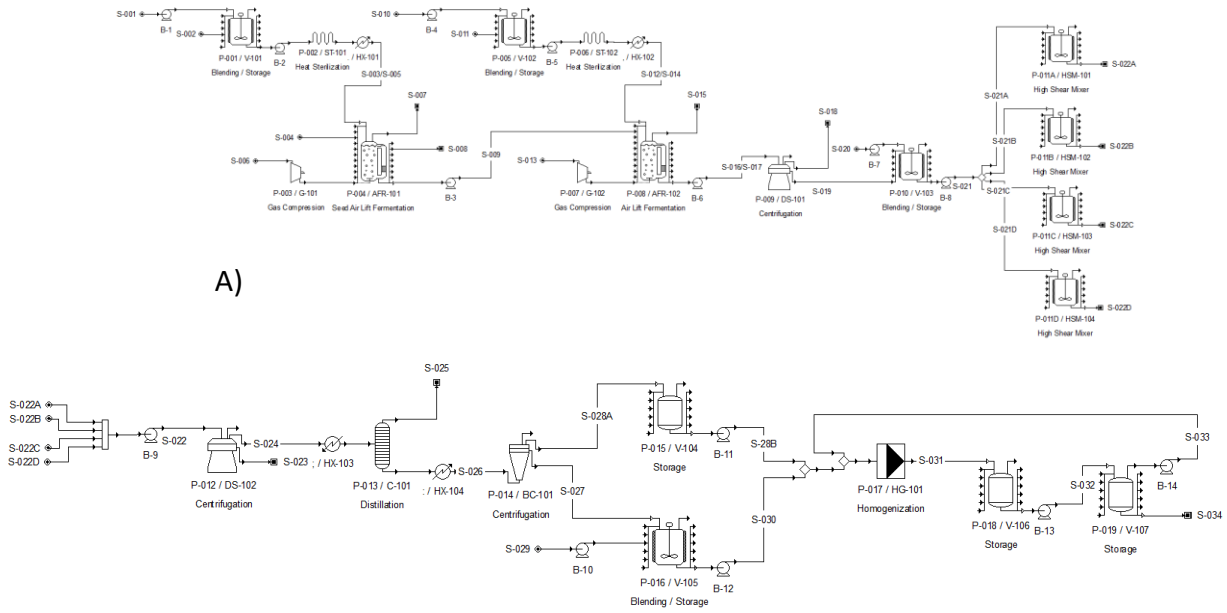


Figure 3. Process Diagram modeled in Super Pro Designer (Version 10) Software

3.2 Major Equipment

Growth Kinetics

The microbial kinetic was designed based on the Haldane substrate inhibition equation with a decay rate and a single substrate (glucose). Kinetic parameters were obtained from Xiao, *et al*. (2024), in which the fermentation performance of a *Thraustochytrium striatum* is described (shown in appendix 10); once these parameters were obtained, the microbial kinetics were modeled in Python (obtained graphics reported in appendix 11). To achieve a high concentration of biomass and avoid substrate inhibition, both airlift bioreactors (seed and main bioreactor) were modeled as a fed-batch process. According to the values provided by the kinetics, it was determined that when the glucose concentration drops to 7 g/L, a feeding must be done to return to 20 g/L of substrate. Therefore, for seed bioreactor and main bioreactor, feed and withdraw rates of 1 L/min and 4 L/min, respectively were established. In that way, the optimal number of feeds to achieve the desired biomass concentration was 2 for the seed

bioreactor and 9 for the main bioreactor. For the seed bioreactor, biomass concentration was 34 g/L, while for the main bioreactor it was 78.18 g/L.

Airlift Bioreactor

Aurantiochytrium sp. requires high oxygen mass transfer for the effective production of primary metabolites during its growth phase. Due to its non-cellulosic and thin cell wall, approximately 2 to 3 nm thick (Marchan *et al.,* 2018), it is particularly sensitive to mechanical shear stress. Consequently, an internal loop airlift bioreactor (ILAB) is employed for its cultivation, as it reduces shear stress and thus improves lipid yield in the biomass compared to stirred tank reactors (Hong *et al.,* 2013; Khanoksinee Sirirak *et al.,* 2021). The ILAB features a vertical cylindrical tank with a gas distributor at the bottom to introduce gas bubbles into the liquid medium, and an internal concentric draft tube that enhances gas-liquid mass transfer and mixing (Khanoksinee Sirirak *et al.*, 2021).

The design of reactor (R-101) and (R-102) are described in Table 3. The systems operate at a temperature of 28°C (Hong *et al.*, 2013) and a superficial gas velocity 0.04 m/s. The volumetric flow rate required will be given with a spider gas distributor with four pipes, each pipe have three lines of holes, each line has 11 equidistant holes with a diameter of 5 mm (Zhong et al., 2022).

Table 3. Physical Parameters of Airlifts Bioreactors.

Description	AFR-101	AFR-102
Operational volume (L)	400	7940
Height to Diameter ratio (H/D)	6	6
Area of downcomer to riser (Ad/Ar)	0.8	0.8
Diameter (D) (m)	0.5	1.5
Height (H) (m)	3	8
Internal Loop Diameter (Di) (m)	0.37	1.12
Area of riser (m2)	0.11	0.98
Area of downcomer (m2)	0.09	0.79
Gas superficial velocity (m/s)	0.04	0.04
Volumetric flow rate (m3/s)	0.0079	0.07
Wall thickness (m)	.005	.005
Material	Stainless steel 316	Stainless steel 316
Sparger	Stainless steel 304	Stainless steel 304

There were several physical parameters considered to optimize de design and functionality of the ILAB, which are discussed in appendix 12

Disc-Stack Centrifuge

Both disc-stack centrifuges (DS-101 and DS-102) were scaled up based on Alfa Laval's CLARA 20 model (Alfalaval, n.d.). Following the heuristic rule $\frac{Q1}{\Sigma 1} = \frac{Q2}{\Sigma 2}$, where Q1 and Σ1 correspond to data given by the model centrifuge, a stream was established to calculate Σ2. As for DS-101, Q2 = 3,031.14 L/h, therefore, Σ2 = 205,855 m^2. On the other hand, for DS-102, Q2 = 2,533.33 L/h, thus, Σ2 = 172,047m^2. The conditions of the centrifuges, shown in table 4, were then adjusted to comply with said value.

Table 4. Technical parameters for disc-stack centrifuges.

	DS-101	DS-102
Discs	150	150
Inner radius of disc	0.089 m	0.089 m
Outer radius of disc	0.18 m	0.18 m
Angle of disc cone	35 °	35 °
RPM	9000	8250

The dimensions and number of discs turned out to be the same for both centrifuges. This will provide an advantage if either one of them happens to break down, as the remaining one could be used for both purposes in the bioprocess, only the velocity would need to be adjusted.

Tubular Centrifuge

The tubular centrifuge (BC-101) was, in turn, scaled-down from Reyes Machinery's GQ75 model. This process followed the same steps as for the disc-stack centrifuges. For BC-101, Q2 = 199 L/h, therefore, Σ2 = 543.43 m^2. The conditions adjusted to comply with this Σ value are shown in Table 5. It is worth mentioning that the formula for Σ depends on the centrifuge type. All calculations can be observed in appendix 13.

Table 5. Technical parameters for tubular centrifuge.

Parameters	BC-101
Radius	0.0625 m
Bowl Effective Height	0.735 m
Gravity	9.81 m/s^2
RPM	5200

High Shear Mixer (HSM)

For the scaling up of the High Shear Mixer the following parameters were considered: 1) Impeller tip speed; 2) The relationship between the rotor diameter and the shear gap distance; 3) Maintenance of turbulent flow; 4) Shear stress. To increase the performance of the system, the following optimal conditions are proposed: 55 °C; 2:1 solvent radio/ wet biomass; impeller tip speed of 11, 509 m/s (Kwak, et al.,2019).

As observed on table 6, the impeller tip speed is maintained on both the bench and industrial scale. Under the reported speed, the HSM suspends wet biomass and is close to maximizing the diffusion efficiency in the extraction system. Note that this is applicable only when a turbulent flow is maintained at an industrial scale. Therefore, Reynolds number calculated at industrial scale is 2.73×10^5, which proves that a turbulent flow is achieved. Lastly, the previous author shares the equation which describes the relationship between shear stress and shear rate for this type of fluid composition (shown in appendix 14), the shear force on the industrial scale is 3.90 Pa, comparable with 4.16 Pa on the bench scale. Turbulent flow is associated with the achievement of an adequate breaking of *Aurantochrytium* cell wall through all the biomass material and facilitating solvent extraction.

Table 6. Technical parameters for High Shear Mixer.

	Bench scale	**Industrial scale**
N (rotational speed, rpm)	7000	1800
Di (Diameter of rotor, m)	0.0314	0.1221
Impeller tip speed (m/s)	11.5	11.5
Shear gap (m)	0.00015	0.0006
Relation (Di/Sp)	209.33	209.33
Reynolds number	6.37×10^4	2.73×10^5
Shear force (Pa)	4.16	3.90
Height of Vessel (m)	-	1.53
Diameter of Vessel (m)	-	0.61

Thirdly, the geometrical relationship between the rotor diameter and the shear gap distance (209.33), on lab and bench scale, this relationship is also used in the scaling up to achieve a similar shear force on the particles and promote the rupture of the cells on the industrial volume.

This small difference on shear stress, together with the optimization of impeller tip speed and turbulent flow, leads us to assume that the favorable conditions for *Aurantochrytium* breakage and squalene extraction are reproduced in the particles on the industrial scale.

$$k\left(\begin{matrix}-1\\ \min\end{matrix}\right) = 9.09 \cdot V \cdot e^{-\frac{4586}{T}}$$

(1)

On the other hand, regarding the process time at industrial level, equation (1), (taking V= as rps and T = temperature in K), this equation was used to relate the lipid extraction rate constant (K, min-1) with the parameters used at industrial level, obtaining a rate of 0.01395 (1/min). Simultaneously, equation (2) in Annexes X. D was used to relate the time necessary to obtain 96 % lipid extraction, considering the rate previously calculated, it being 3 hours and 9 minutes.

$$\ln\left(\frac{Rm}{Rm - R}\right) = k \cdot t \qquad (2)$$

HSM geometrical characteristics

In order to obtain the dimensions of the HSM, we considered what was described by Vashisth, V., Kumar, V., (2022), and Doran (2013) concerning the heuristic rules that allow relating the impeller diameter and the tank diameter, described in table 5, thus we obtained an operational volume for each of the HSM of 379.88 L. To satisfy the total inlet volume to the HSM per batch, 4 HSM are used simultaneously on the first batch for 3 hours and 9 minutes, for the second batch, 3 HSM are used simultaneously for 3 hours and 9 minutes, giving a total of process time of 6 hours and 18 minutes. The equipment will be personalized for the dimensions previously mentioned.

Flash distillation

A flash distillation unit will be used to remove the ethanol solvent employed in the lipid extraction process. This method is suitable due to the significant difference in the boiling points of the components: ethanol at 78.4°C and squalene at 421.3°C, along with water and other lipids. Accurate thermodynamic data are crucial for the proper design and analysis of distillation columns, as equipment often fails to meet performance specifications due to a lack of such data. To address this, the chemical process simulation software Aspen Hysys® was utilized for its simplicity and reliable results in obtaining the vapor-liquid equilibrium (VLE) curve. The simulation was conducted at a temperature of 85°C and a pressure of 1 atm, results of each stream are shown in appendix 15.

To design the physical dimensions of a flash drum, it is essential to know the separation factor, inlet flow, and molar compositions of each phase after the heat exchanger. With the vapor and liquid

streams and their compositions known, the calculation for the dimensions of the distillation drum begins by determining the flow rate ratio (Flv) and the empirical constant of Souders–Brown (Ks). This parameter allows us to determine the permissible vapor velocity (Up) in the region of maximum cross-sectional area, and therefore the height and diameter dimensions. Ensuring that the drum provides sufficient disengaging space to allow the vapor to separate from the liquid is crucial (Wankat, 2008; Blackwell, 1984; Rodelo *et al.*, 2019).

Table 7. Parameters for flash distillation C-101

Parameters	P-013 / C-101
Height to Diameter ratio (H/D)	3
Diameter (m)	1.5
Height (m)	4.5
Height from the feeding port to the top of the drum (hv) (m)	1.7

The change in temperature is achieved with a heat exchanger reaching 85°C in the stream, there is no pressure dropped considered.

3.3 Minor Equipment

To comply with the necessary temperatures, stream velocities, and air flow needed by the major equipment, pumps, compressors, heat exchangers and even tanks were essential. This bioprocess requires 14 pumps at different points. After evaluating the necessary flow velocity at every point, a pump with a maximum flow of 6,000 L/h was chosen from the provider SODIMAC.

The bioprocess only needed two air compressors: one for the seed airlift bioreactor and another for the big airlift bioreactor. These were chosen based on the necessary air flow of 3,806,054 L and 30,675,334 L, respectively. Their energy consumption was calculated based on their respective motor power, time, and efficiency of 85 percent (Air Supply UK, 2023).

To achieve a sterilization level of 1 spore per 1,440 hours of continuous sterilization flux for the seed and production bioreactor, the medium requires retention times of 1.51 minutes at 116.21°C and 2.35 minutes at 111.48°C, respectively. At the same time, based on the calculations in appendix 13 the heat exchangers net satisfies a UA (overall transfer coefficient * area) of 3.46 and 8.68 kW/°C for the seed and production reactor respectively.

Based on the energy requirements for the bioprocess, a boiler with a capacity of 910.42 kW is necessary to satisfy the largest exchanger, assuming the steam exchangers do not operate simultaneously, as shown in the Gantt chart (Fig.4). The total energy required per batch is 2,269.41

kWh to satisfy equipment ST-101, ST-102, HX-103, HSM, and V-105. Additionally, a water chiller with a power of 601.25 kW is needed to meet the requirements of equipment HX-101, HX-102, and HX-104, with a total energy requirement of 642.83 kWh.

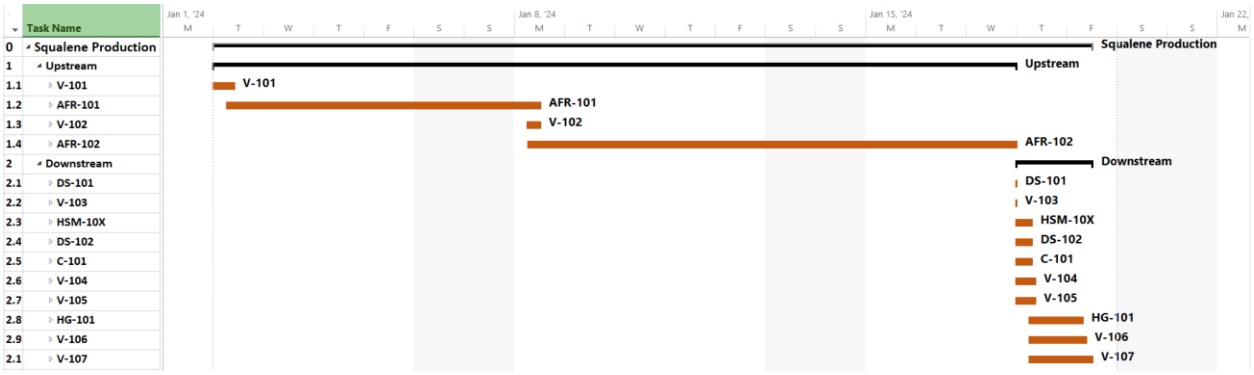
Between major operations, tanks for storage and/or mixing of the effluents must meet the next operation's parameters. The dimensions of the tanks and the energy required for mixing are reported in appendix 13.

A total of 123 meters of austenitic 316L stainless steel pipes are needed. This already considers the 30 meters needed for the sterilization retention time for the media at the seed and big airlift, with an inner diameter of 0.05 and 0.1 meters, respectively. The chosen material has high sanitary applications: it does not rust, it's not reactive nor additive, nor absorptive, and so the streams are never compromised (González, 2001). Also, seven accessories were estimated, all being valves at critical points.

3.4 Process logistics (Gantt chart)

To ensure a well-coordinated process, a Gantt chart was created considering every unit operation and its CIP and SIP time, respectively.

Figure 4. Gantt Chart of total process modeled in Microsoft Project.



Gantt_squalene_bioprocess.mpp

3.5 Plant Layout

To ensure an efficient workflow and minimize the risk of cross-contamination, the layout follows a linear flow of production, with 1365 m^2 as shown in Figure 5. The production equipment is arranged in a sequence that allows for the continuous movement of materials from one stage to the next without backtracking. Machines and workstations are marked with a specific physical space, known as static

surface area (Se). Additionally, extra space, known as gravitational surface area (Sg) (circular area), is reserved to allow operators to perform their tasks and to accommodate materials and tools. Furthermore, an evoclution surface area (Sev) is required to facilitate the movement of materials and operators (marked as the minimum space necessary with dotted line).

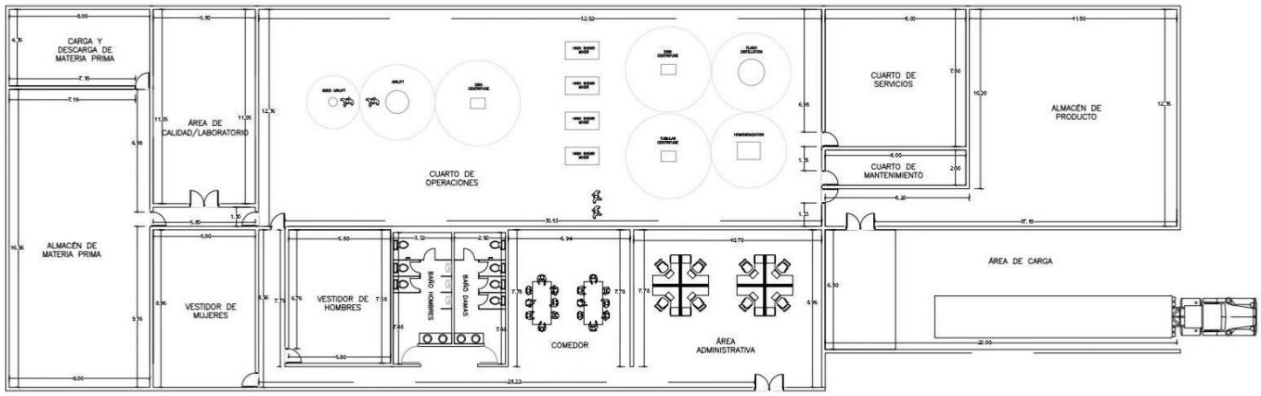


Figure 5. Design of plant layout

4. Security, quality, and environmental aspects

4.1 Legislative framework

Mexico has several regulations to comply with to produce and distribute the bioproduct in question. Starting with the purity of the squalene, this should be of at least 70% according to the FDA, which is why AquaVit has a purity of 81%. The bioprocess should follow the good manufacturing practices (GMPs) established by NOM-259-SSA1-2022. These include wearing the right protective clothes, and being clean, while working in the production area; using clean water that is periodically verified for the allowed limits of chlorine and solids; have a system of evacuation of residual water; and more. The DOF Agreement of 21/05/2010 states the prohibited and restricted substances in the elaboration of cosmetic products, among which is chloroform. Although this substance is usually used for the extraction of lipids, this bioprocess avoids it completely and instead uses ethanol. As for the ethanol, NOM-076-SSA1-1993 states that high precautions must be taken in the form of having a fire extinguisher handy, the use of goggles and gloves, storing it away from oxidants, and having to take blood samples from personnel to ensure their health.

To ensure the harmlessness of the product in terms of microbial content, NOM-089-SSA1-1994 provides many protocols for the identification of many different organisms. In the case of *Salmonella* sp., for

example, depending on the agar they grew, their morphology will be different if present. The goal is to rule out any contamination by microbes, so these protocols are to be carried out regularly.

Given that the plant also counts with a laboratory for the verification of quality and harmlessness of the product, NOM-059-SSA1-2015 should be followed. This regulation details the necessary equipment, the adequate uniform for personnel, good manufacturing and storing practices, and qualification and validation of personnel and equipment.

The final product must have a label according to NOM-141-SSA1/SCFI-2012, where the place of production, batch and expiration date must be mentioned, the contents must be listed from highest to lowest, the instructions of use must be in Spanish, and total quantity of the product.

Finally, when it comes to handling residues, there are also important regulations to keep in mind. According to NOM-001-SEMARNAT-2021, there are limits for contaminants allowed in the discharge of residual waters. In the case of marine zones, the daily average can be 18 mg/L of oil, 24 mg/L of total suspended solids, 25 mg/L of carbon, 30 mg/L of nitrogen, 18 mg/L of phosphorous, and a pH range from 6 to 9. Furthermore, given that the law of biosecurity of genetically modified organisms does not classify mutagenesis as a tool for GMOs, there is no risk of sanctions for an accidental release of this microorganism to the environment.

4.4 Residue Disposal

In accordance with the legal framework previously described, reverse osmosis will be performed on the residual saline water to reduce it within the permissible limits, according to Wenten, I., (2016) this is a recommended practice at the industrial level. Simultaneously for the disposal of carbon dioxide, a strategic partnership with CEMEX, through its Carbon Capture, Utilization and Storage (CCUS) initiative, in this initiative the CEMEX capital venture supports startups seeking to approach a low carbon economy through CCUS. For ethanol, this should either be evaporated (if it's small quantities) or incinerated, according to UNAM (2011).

According to Dillon, (2020) and the definition of NOM-087-ECOL-SSA1-2002 regarding a biological-infectious risk agent, cellular debris not considered as a biological-infectious risk, considered an organic waste, so the recommendations of EPA (2020) regarding its collection and transportation will be followed.

4.2. Product validation

There are two very important characteristics the product should comply with to ensure its stability: presence of antioxidants and a particle size of 20-200 nanometers (Chellapa et al., 2016). While a considerable amount of antioxidants prevent the oxidation of the product for 12 months (Rastrelli, et al., 2002), a particle of said size confirms a successful nanoemulsion, which in turn also aids in oxidation prevention. The presence of antioxidants must be quantified using HPLC and the protocol to follow is as established by zanova, et al. (2016). To determine the particle size, a Malvern Zetasizer Nano ZS is used per Peng et al.'s instructions (2019).

The quality of the product heavily relies on the purity of squalene. To verify a purity of 81%, an assay with HPLC should be performed based off Liu et al.'s (2021) protocol on the matter. Also, applying for a COSMOS Certified for raw materials guarantees this product is not only eco-friendly, but also compatible with human health.

4.3 Applicable pre-requisite programs

In agreement with the previously mentioned legal framework and the good manufacturing practices for cosmetic products described in NOM-259-SSA1-2022, the potential hazards for the safety and effectiveness of the AquaVit product were determined, as described in appendix 16. In accordance with the hazard analysis and critical control point (HACCP) methodology, 7 control points were determined, together with 4 operational prerequisites, described in appendix 17. The control points and prerequisites #1 during fermentation are associated with maintaining the safety and productivity of the system. Simultaneously, CCP #5, #7, PPRO #1 are associated with preserving the suitable characteristics of the product for its effectiveness. Finally, the use of a doble-checking system of inspection of raw materials and packaging material, in conjunction with twice-monthly training of personnel in good manufacturing practices, was identified as a necessity.

4.5 Sustainable Development Goals

The squalene production plant aligns with the Sustainable Development Goals (SDGs) and the Agenda 2030 in Mexico, particularly Goal 9, which aims to build resilient infrastructure, promote sustainable industrialization, and foster innovation. One of the project's main objectives is to achieve sustainable industrial development and technological progress. The sustained growth of personal care products and squalene-based items involves creating an industry that supports technological innovation,

such as the use of biotechnological products. Although these require investment and research, they offer a sustainable alternative for the manufacturing industry in terms of providing local raw materials.

Consequently, Indicator 9.3.1 is crucial for us as a company, as it focuses on promoting sustainable industrialization and fostering innovation in small industries and other enterprises in developing countries like Mexico. This indicator provides a tangible metric to evaluate the added value in the development of our product as a market value addition and its integration into supply chains. It also conceptualizes the development of a biotechnological plant in the country and the accessibility to financial services.
Furthermore, the company aims to indirectly impact Indicator 9.2.1.a, which measures the manufacturing value added as a proportion of GDP. We seek to promote sustainable industrialization that enhances the contribution of the manufacturing industry to the Gross Domestic Product (GDP).

5. Economic Assessment

5.1 Investment Cost

To assess the financial requirements for the successful launch and operation of our enterprise, an analysis of total direct and indirect costs was performed. This economic assessment aims to provide and ensure an efficient production with safety and quality standards; a detailed overview of the costs associated with the establishment of the plant is discussed. Established methodologies from the book “Plant Design and Economics for Chemical Engineers” were used to estimate costs of the plan design.

The total capital expenditure (CAPEX) was calculated by summing the costs of major equipment, minor equipment, and miscellaneous items. Major equipment necessary for the production process cost $3,424,534.32 MXN, while minor equipment was estimated at $893,384.17 MXN. Additionally, miscellaneous costs, which include quality control equipment, office furniture, and land acquisition, amounted to $1,960,336.16 MXN. The total CAPEX thus combines these expenditures. Beyond the initial equipment costs, additional direct costs were estimated based on standard percentages of the total CAPEX. The installation of purchased equipment was calculated at 30% of the total CAPEX, ensuring proper setup and integration of the machinery. Instrumentation and controls, essential for monitoring and managing the production process, were estimated at 8% of the total CAPEX.

Furthermore, electrical installation costs, crucial for providing the necessary power infrastructure, were calculated at 2% of the total CAPEX.

Vitality´s total indirect costs represent 25% of the total fixed capital, estimated at $1,575,412.66 MXN, which includes key elements necessary for the successful establishment and operation of our facility. This includes a) Plant Development: $252,066.19 MXN for planning, design and engineering services to ensure the facility meets all operational and regulatory standards; b) Contingencies: $945,248.29 MXN designated for unforeseen expenses or potential project overruns; C) Contractors and Construction Management: $378,099.28 MXN for contractor fees, construction management, and labor to build the production facility. Acquisition of land is expected, therefore settling in an industrial building with existing infrastructure will facilitate a cost-effective setup.

A mortgage interest rate of 9.25% was considered for this project. The rate was obtained from CitiBanamex, since it was chosen based on its competitive interest rates and favorable credit terms, which are essential for project financing.

The total fixed capital investment, which includes both direct and indirect cost segments, was calculated to be $10,397,730.18 MXN. Total calculations are reported in appendix 18.

5.2 Financial Sources

One of the main sources of financing for biotech ventures is investment funds Innpactia, which is the biggest one in Latin America for companies with a sustainable purpose. Simultaneously, the multilateral investment fund, managed by the Ibero-American Development Bank, is a key partner, since is a viable source of financing in Latin America for innovative ventures in line with sustainable development. It is proposed that this type of source of financing contributes 15% of the initial investment amount.

Furthermore, being a technological innovation project that promotes sustainability, it is possible to access convocations related to research, such as the program of stimuli for research, development and innovation by the government of Mexico. It is worth mentioning that a free process of National Registry of Scientific and Technological Institutions and Companies (RENIECYT) must be carried out prior to the call for proposals, this incentive covers up to 30% of the company's initial investment. However, due to the required amount of initial investment, a credit loan for entrepreneurs from Banamex bank with an interest rate of 9.25 % must also be considered.

5.1 Assessment of Economic Sceneries

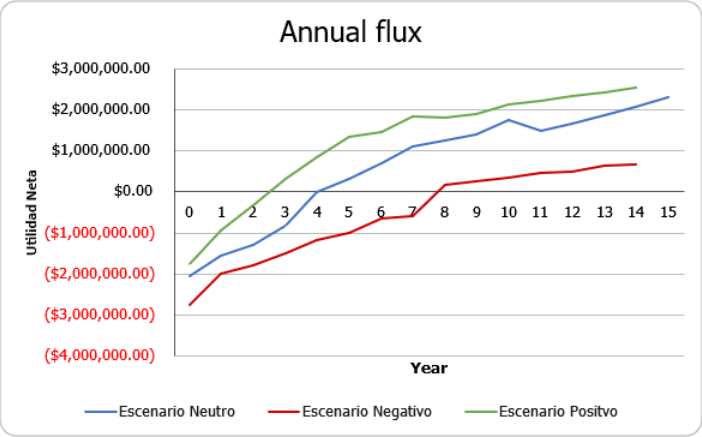


Figure 6. Annual Flux.

According to figure 6, an approximation was made regarding a positive scenario with a profit margin of 28% for the first year. It should be emphasized that this margin is representative, since the market price is $2,500 MXN, while the annual production cost of Aquavit is $1,018 MXN per unit, which allows a positive net profit for the second year in the positive scenario. On the other hand, the neutral scenario generated a more representative approach, since for the fourth year it allows the company to have a net profit, with a marginal contribution of 20% for the first year. Finally, the negative scenario runs the time in which a net profit is observed until the eighth year, considering a marginal contribution of only 12%.

Based on the three generated scenarios, the Internal Rates of Return (IRRs or TIRs) obtained were –15%, 11%, and 15% for the pessimistic, neutral, and optimistic scenarios, respectively. From these results, it can be observed that the project is not profitable in a pessimistic scenario, representing a high-impact risk to consider during its implementation and development. However, the IRRs presented in the neutral and optimistic scenarios indicate a very attractive return on investment that could attract investors and facilitate the project's development and growth. Considering the different marginal contributions of the project and based on the generated scenarios, it is recommended to conduct a market study to evaluate the product's selling price.

5. Conclusions and recommendations

This project proposes a sustainable production of squalene from *Aurantiochytrium sp*. Yonez 5-1, for use in the cosmetic and personal care industries as an active ingredient or component for

cosmetic formulations. Vitality positions itself as an innovative alternative for cosmetic manufacturers in Mexico, in which not only meets market demands for hydrating and anti-aging formulations but also ensures greater stability, bioavailability, and feasibility at industrial scale. Our project demonstrates great potential for being profitable and sustainable. Furthermore, it aims to operate in alignment with the Sustainable Development Goals (SDGs) by focusing on innovation, sustainability, and social responsibility, we believe this project can set new standards in the industry and contribute positively to both the market and society.

6. Appendix

Appendix 1

***UV mutagenesis for Aurantiochytrium sp.* Yonez 5-1**

Microbial Cultivation

The methodology employed was based on that described by Liu, et al. (2020). The procedure is conducted at the facilities of the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, headquarter Guadalajara. Aurantiochytrium sp. Yonez 5-1 was purchased from Científica Senna from Ciudad de México. Strain was inoculated into M4 liquid medium made with 100% filtered natural seawater containing glucose (2%), yeast extract (0.10%), tryptone (0.15%), and KH2PO4 (0.025%) (Yaguchi, et al., 1997). The seed inoculum of Aurantiochytrium sp. Yonez 5-1 was cultured in a shaking incubator at 23°C and 180 rpm for 48 h. In a 250 mL flask, 100 mL of medium were inoculated with 5 mL of the above culture. Three biological replicates of each sample were examined (Liu, et al. 2020).

UV-Mutagenesis

Before UV mutagenesis, the UV crosslinker was turned on for 30 min to stabilize the light waves. The microorganism solution was diluted 105 times and applied to the plate. Then the microorganisms on the plate were mutagenized after 24 h of incubation in a constant-temperature incubator at 23°C. The plates were placed at 50 W UV crosslinkers and irradiated for 30s. After mutagenesis, the plates were incubated for 48 h in the dark, and then, the number of colonies was counted, and the lethality was calculated (Liu, 2020). Under the described conditions, the survival rate is 83.67% (Liu, et al., 2020).

Screening of the mutant strain

After UV irradiation, approximately 135 colonies were obtained from the surviving cells. Mutant screening was based on dry cell weight (DCW) enhancement. A total of 20% of the colonies exhibited

significantly enhanced cell growth compared with the parent strain. To verify the hereditary stability of the mutants, 5 of them were selected and cultivated continuously in a shake flask for ten generations. The fermentation conditions during continuous subculture remained the same and were set as: inoculum 10% (v/v), culture temperature 23°C, initial pH 6.5, and fermentation medium volume 1000 mL. As reported by Liu, et al. (2020), there was no significant difference for biomass production observed among the ten generations. The results showed that UV irradiation (50 W, 30 s) could be utilized as a breeding strategy to screen enhanced strain in Aurantiochytrium sp. (Liu, et al., 2020).

Appendix 2

Appendix 2. Aquavit final product presentation

Appendix 3

General information	Product Description
Product name: AquaVit **Technical Name:** Oil-in-water 81% pure squalene nanoemulsion **Scientific name:** 2,6,10,15,19,23-Hexamethyltetracosane (API) **CAS:** 111-02-4 **Formula:** $C_{30}H_{50}$ **Country of origin:** Mexico	**Expression system:** *Aurantiochrytium* sp. Yonez 5-1 **Density aprox. (g/L):** 951.72 **Purity:** ≥80% **Solubility:** ≤ 1 mg/mL **Physical form:** Emulsificated oil **Color:** Clear **Odor:** Odorless **Presentation:** 5 L polypropylene buckets

Commercial Information	Distribution method
Packaging: 2.5 gallons polyethylene vacuum sealed buckets. **Units per box:** 10 units	**Storage temperature:** 15 – 25 °C **Sales:** Direct to business.
Ingredient List	**Processing Methods**
Water 66.82% Squalene 22.17% Soybean Lecithin 2.61% Antioxidants 2.56% Lipids (TRG+STER+PHOSP) 1.18% Ethanol 0.51%	After a period of fermentation, microalgae are harvested through centrifugation before undergoing lysis with ethanol in a high shear mixer. The resulting cell debris and remaining water are centrifugated out, and the supernatant goes through a distillation column to remove the ethanol from the oil. Finally, the oil undergoes a nanoemulsion in a homogenizer, and the final product is achieved.
Intended Use	**Type of Consumer**
Ingredient to be added to cosmetic formulations for anti-aging, hydrating, and/or antioxidant properties.	Cosmetic businesses that produce anti-aging, hydrating, and/or anti-oxidating products; either with squalene in their formulation or with an interest with adding it in.
Additional Information	
Document creation date: May 25, 2024 **Last review date:** May 25, 2024 **Company contact:** 815-965-3311	

Appendix 4

Appendix 4. Decision Matrix for Plant Location.

Factors	Weight	Monterrey	Edo Mex	Querétaro	Jalisco
Raw Materials (Saline wastewater)	20	Micro manufacturers of dairy products.	Close to major dairy product manufacturers.	Small dairy product manufacturers.	Major manufacturers of dairy products.
Market	25	Less accessible to most cosmetic manufacturers	Close to manufacturers	Industrial growth	Close to manufacturers
Climate conditions	10	Max. 36°C | Min. 10°C	Max. 28°C | Min. 3°C	Max. 30°C | Min. 6°C	Max. 32.9°C | Min. 7°C
Availability of water	10	Medium-high risk [1]	High risk [2]	High risk [3]	Medium-high risk [4]

Workforce	15	N/A	6.15 K [5]	3.66 K [6]	8.36 K [7]
Land cost	10	$108 m^2	$31.19 m^2	$80 m^2	$95 m^2
Protection against natural events	5	Low – High	Very low – High	Low – High	High – High

Appendix 5

Appendix 5. Decision Matrix for technology selection for squalene extraction.

Extraction Technology	Technological Facts	Raw Material	Development Time	By-products and Residues	Costs	Yield
Supercritical CO_2	Supercritical CO_2 behaves as a nonpolar, lipophilic solvent suitable for the extraction of hydrocarbons (Yildiz, *et al*., 2017). - Production of solvent-free crude lipids (Halim, *et al*., 2021). - Non-toxic, non-flammable (Yildiz, *et al*., 2017). -Reduces thermolabile degradation in extracted	-Dry Biomass (preferably lyophilized). -CO_2 as Solvent (99%) -Ethanol as co-solvent. - Supercritical Fluid Extraction Equipment (TOP 121-40-24) which has high industry production (ToptionLab, 2022)	TLR 9: System successfully tested in industrial scale, proving that it is highly scalable. It has been successfully applied as a separation and fractionation technique for obtaining squalene from dry biomass.	-Residues: Residual biomass: thus, it may contain proteins and carbohydrates from the microalgae that can be recovered and used in the food industry, or as fertilizer (Halim, *et al*., 2021). Co-solvents: Ethanol disposal must be carried out according to regulations. Solvent (CO_2): The	High investment capital for the extraction equipment (Halim, *et al*., 2021). High costs are because pure CO_2 is required. However, it can be captured and adapted to a circular economy model for recirculation or sale as a by-product (Rosales, *et al*., 2017). High energy requirement	Due to its intermediate liquid-gas properties, it can penetrate through cellular matrices rapidly and produces a higher total lipid yield (≥95%) (Halim, *et al*., 2021). Squalene

	compounds (Rosales, *et al*., 2017). - Achieves an extract with high purity (Rosales, *et al*., 2017). -Requires biomass drying previously (Calvo, 2010).			technology allows recirculation of the fluid. However, it needs to undergo certain purification processes to remove any residual impurities (Vázquez, 2008). Filters to purify CO_2. -By-products: Antioxidant compounds (such as carotenoids or astaxanthin) Pigments. Extraction can concentrate compounds that are not lipids but have high commercial value in the cosmetic	which means elevated costs. Initial investment of equipment approximately ranges from 1.5 million to 5 billion USD.	

				industry (Vázquez, *et al*., 2007).		
Organic solvent extraction (Hexane and methanol)	- Most common method used as standard and extract is considered 100 % of extractable Matter (Rosales-García, *et al*., 2017). - Hexane is typically the solvent used for large scale extractions due to its relatively low cost and high extraction efficiency (Rosales, *et al*., 2017). -Selectivity is not easily tuned when non-polar organic solvent is used, only	-Dry Biomass (consider the energy and equipment required for drying). - Hexane and methanol as solvents (Chanioti & Tzia, 2020). -Vacuum evaporator for solvent extraction (Chanioti & Tzia, 2020).	TLR 8: Complete successfully tested in industrial scale, proving that it is highly scalable. Nonetheless, it is the most common option in lipid extraction, and has become an obsolete process. More advanced extraction methods have been developed (Gong, *et al.*, 2017).	-Residues: Hexane and chloroform; solvents are usually evaporated (Patel, *et al*., 2020). Solvents need to be classified as chemical residue. Residual biomass: After extraction Requires treatment to remove any residual solvent prior to disposal (Correa, *et al*., 2020). -By-products: During extraction, not only lipids are extracted, but also other solvent-	The approximate costs may vary depending on several factors, such as the scale of production, however an approximate price can be estimated according to literature. Costs between $50,000 and $500,000 USD or more per year have been reported (Ramluckan, *et al*., 2014). If solvents are purified and recirculated, the cost can be lower.	Massetti *et al.* results suggest that it is possible to achieve 96.3% of lipids extraction yield from microalgae at industrial scale (2019). Most of the lipids (60-70%) can be extracted within the first eight hours (Gong, *et al.*, 2017).

	limited amount of neutral lipids can be extracted (Halim, *et al*., 2021). - Lipid extraction rate is slow and lipid extraction requires a long time for completion (Halim, *et al*., 2021). - It consumes low energy as lipid extraction is conducted near ambient conditions. However, organic solvent needs to be removed from the lipids via energy-intensive liquid–liquid separation method			soluble compounds such as pigments, waxes, and sterols. Metabolites can be recovered as they have a high value in the cosmetic and food industry (Chanioti & Tzia, 2020).		

	(Halim, *et al*., 2021). - Toxic organic solvents are used. Residues of hexane can be toxic for cosmetic purposes. -The use of hexane raises several environmental concerns, especially for industrial scale. -It's reported that it may cause lipid oxidation (Correa, *et al*., 2020).					
High Shear Mixer (HSM) with Ethanol Extraction	- New method to reduce the energy and solvents required for lipid extraction (Kwak, *et al*., 2019). - Suitable to treat wet microalgal	- Wet biomass: studies have shown similarly, even higher extraction yields with wet biomass compared to dry	TLR 8: Complete and certified system through tests and demonstration. It is proven to be highly scalable, however, there´s	Residues: -Ethanol: In distillation, ethanol can be captured, purified and recirculated. If it is not possible to recirculate it, the regulations	High Shear Mixer can be applied to the downstream process with low energy consumption and solvent usage, thereby dramatically improving the	Kwak, *et al*., reported a 90% lipid extraction yield achieved with the lowest energy consumption and solvent

	biomass for efficient cell disruption and lipid extraction (Kwak, *et al*., 2019). - No prior drying required (Kwak, *et al*., 2019). - Widely used due to the ease of alcohol removal by evaporation. - High scalability and compared to other mechanical disruption method (Lee, *et al*., 2012) -Due to ethanol low selectivity, there are many unwanted impurities in extracted oil such as	biomass in a HSM (Kwak, *et al*., 2019) - High Shear Mixer industrial equipment. -Ethanol as a solvent.	opportunity for optimization. Technology meets industry standards.	for its disposal must be followed (Kwak, *et al*., 2019). -Cell debris: Contains proteins and carbohydrates from the microalgae that can be recovered and used in the food industry, or as fertilizer (Halim, et al., 2021). By-products: -Pigments: Provide stability to the final product and confer an added value due to their antioxidant properties (Winwood, 2013. - Phospholipids: Used as	overall economic feasibility compared to the conventional process (Kwak, *et a*l., 2019). Initial investment varies widely and must be evaluated based on specific process requirements and equipment characteristics. The investment average ranges from 150,000 USD to 300,000 USD (Ginghong, s.f.).	usage in a high shear for lipid extraction (2019).

	phospholipids (Winwood, 2013). -High Shear Mixer has a very high potential for cell disruption and lipid extraction (Kwak, *et al.*, 2018).			emulsifier, solubilizer and hydration agent. Confer added value to final product. (Groisman, 2014).		

Appendix 6

Mass Balance Seed Airlift Bioreactor and Main Airlift Bioreactor

Appendix 7

Global Mass Blance

Table 2. General mass balance to produce an O/W squalene nanoemulsion.

Component	Inlet (kg/batch)	Outlet (kg/batch)
Biomass	13.54	23.53
Glucose	1,140.61	31.79
Water	13,036.75	13,612.16
Tryptone	75.008,273.03	8.84
Yeast Extract	31,122.37	0.00
Terbinafine	0.0037	0.0037
Oxygen	0.00	8,206.81
Nitrogen	1,200.34	31,122.37
Carbon Dioxide	0.00	206.5
Ethanol	0.00	1,200.34
Carbohydrates	0.00	40.50
Proteins	0.00	67.501
Phospholipids + glycolipids	0.00	7.49
Non-Polar Carbs + Proteins	0.00	4.08
Squalene	0.00	287.51
Triglycerides	0.00	6.12
Sterol Steres	0.00	1.68
Pigments	0.00	33.21

Cell Debris	0.00	18.39
Soybean Lecithin	29.0	28.97
Total	54,906.74	54,908.24

Appendix 8

As observed in the present document, different methodologies were followed to calculate the required energy for the bioprocess. When it comes to the major equipment, the motor power, which was taken from a reference on the market specific to each equipment, was divided by the reported efficiency and then multiplied by the working hours to calculate the actual energetic consumption. This was the case for every piece of equipment except the distillation column, whose energetic consumption was modeled in Aspen software; and the high shear mixer, whose calculations can be observed in Energy Balance - Calculations. The energy required for the seed airlift and main airlift was a special case, given that the actual tank does not require energy. Rather, the air compressors that supply the necessary air flow do. These calculations can be observed in the same tab as the mayor equipment.

The calculations for the minor equipment were even more varied. For the pumps, the flow velocity at each one was calculated based on the required time a certain volume had to be transported in, or sometimes the time was calculated based on the total volume and flow velocity. The former case was true for equipment such as the seed and big airlift bioreactors, where the time was already fixed because of the microorganism's kinetics. Also, for any other occasion where the flow could be adjusted to be as quick as possible to economize on time, such as the input of ethanol in tank V-103. The latter case applied for centrifuge DS-101 and the homogenizer HG-101, where the flow was already pre-established. According to Pumps & Systems Magazine (2020), the type of pump chosen has an energetic efficiency of 75 percent. The motor power of every pump (0.7355 kW) never varies thanks to them being the same make and model, so the energy consumption depends on the efficiency and time of work.

To obtain the necessary energy for the impellers of the mixing tanks, a Rushton impeller was proposed due to the fluid's low viscosity. The tank dimensions were set with a height-to-diameter ratio (HL/DT) of 1.00 and an impeller-to-tank diameter ratio (Di/DT) of 0.33. The rpm selection ensures turbulent flow based on the Reynolds equation (Equation 1). From the calculated Reynolds number, the power number is determined using the general relationship between power number and Reynolds number as presented by Doran (1995). The required power is then calculated using Equation 2.

$$Re = \frac{Du\rho}{\mu}$$

(Equation 1)

$$P = N_P \rho N_i^3 D_i^5$$ (Equation 2)

Energy requirements in the sterilization cycle

To achieve the temperature necessary for producing 1 spore every 60 days of operation during each sterilization cycle of the medium, we need to consider the acceptable number of cells (N2/N1), the linear velocity of the flow, and the Reynolds number (Equation 1). Using the relationship between the axial dispersion coefficient and the Reynolds number as presented by Doran (1995), we calculate the Peclet number (Equation 3). From the relationship between the Peclet number and the acceptable number of cells, we determine the Damköhler number and calculate the death constant kd (Equation 4). Based on kd , we then determine the required temperature (Equation 5).

$$Pe = \frac{uL}{\mathscr{D}_z}$$ (Equation 3)

$$Da = \frac{k_d L}{u}$$ (Equation 4)

$$T = \frac{\left(\frac{-E_d}{R}\right)}{\ln\left(\frac{k_d}{A}\right)}$$ (Equation 5)

Heat exchangers

For the heat exchangers, the energy required for the process was obtained from Equation 6 based on the difference of temperature in each equipment.

$$\hat{M}_h C_{ph}(T_{hi} - T_{ho}) = \hat{M}_c C_{pc}(T_{co} - T_{ci}) = \hat{Q}$$ (Equation 6)

Energy Balance - Calculations

Appendix 9

Medium Formulation was obtained from the standardized medium for *Aurantiochytrium* sp. (ATCC, 2020)

Appendix 9. Medium Formulation for *Aurantiochytrium* sp. Yonez 5-1

Components	Concentration (g/L)
Yeast Extract	1.0

Tryptone	10.0
Glucose	20.0
Terbinafine	0.01
Artificial Seawater	1.00 (L)

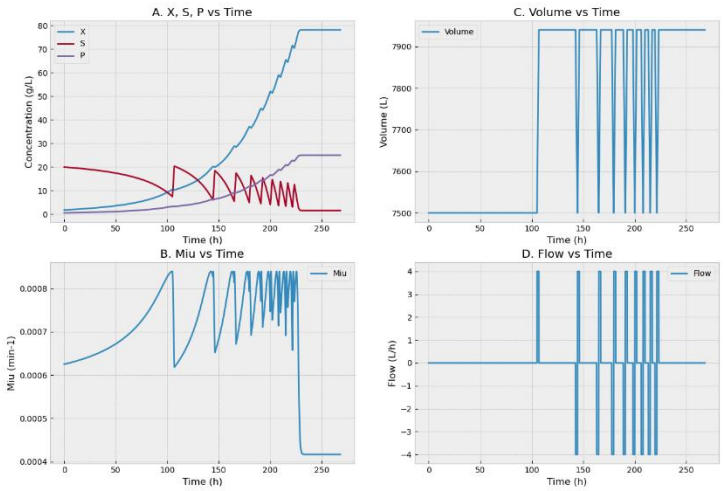
Artificial Seawater will be made by adding 37.0 g of natural sea salt (Sal Real de Colima). Bring final volume up to 1.0 L. For each 900 mL of artificial seawater add each of the above components in the order indicated. Adjust the final volume to 1.0 L.

Appendix 10

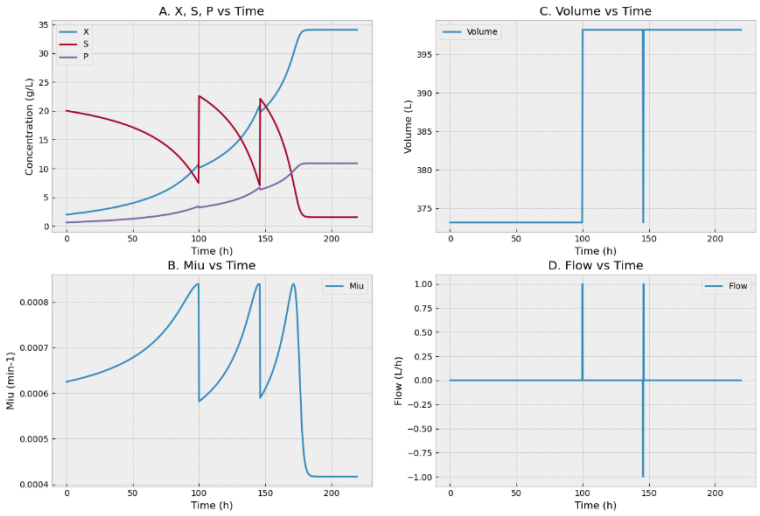
Table 6. Kinetic Parameters for *Aurantiochytrium sp*.

Kinetic Parameter	Value
Maximum Specific Growth Rate (µmax)	0.147 h^{-1}
Substrate Saturation Constant (Ks)	7.19 g/L
Death Rate Constant (kd)	0.025 h^{-1}
Yield Coefficient of Biomass on substrate (Y x/s)	0.696
Inhibition constant (ki)	7.81 g/L
Yield Coefficient of product biomass (Y p/x)	0.32

Appendix 11



Appendix 11.1. *Aurantiochytrium sp.* Yonez 5-1 growth kinetics in main Airlift Bioreactor. A) Kinetic graph of biomass (X), substrate (S), product (P) over time. B) Volume of the bioreactor over time. C) Specific growth rate over time. D) Flow of the feed over time.



Appendix 11.1. Graphics of *Aurantiochytrium sp.* Yonez 5-1 growth kinetics in seed Airlift Bioreactor. A) Kinetic graph of biomass (X), substrate (S), product (P) over time. B) Volume of the bioreactor over time. C) Specific growth rate over time. D) Flow of the feed over time.

Appendix 12.

There were several physical parameters considered to optimize de design and functionality of the ILAB (Benthum *et al.,* 1999; Guieysse *et al*., 2011; Euzen *et al*., 2004; Chisti & Moo-Young, 2003; Sikula *et al.,* 2007): Height to Diameter Ratio (H/D): The H/D ratio of 6 was chosen based on several key considerations to optimize oxygen transfer and liquid circulation within the bioreactor. Increasing the height of the fermenter enhances the partial pressure of oxygen at the base, providing a larger driving force for oxygen transfer, which is essential for maximizing mass and oxygen transfer efficiency. Research indicates that an H/D ratio greater than 4 to 5 effectively limits end effects, such as those from the distributor and the upper portion of the fluid medium and reduces liquid recirculation. Additionally, ensuring a sufficiently tall (greater than 2 to 3 meters) and slender column supports these benefits.

The ratio of the cross-sectional area ratio of the downcomer to the riser (Ad/Ar): This is a crucial parameter for optimizing mass transfer in an internal loop airlift bioreactor (ILAB). This ratio significantly influences the liquid circulation and mixing time within the reactor. Ratios between 0.6 and 0.8 are considered optimal for performance, so a ratio of 0.8 was chosen based on references indicating it as ideal for maximizing mass transfer efficiency. The gentle fluid movement created by the difference between the average gas holdups in the riser and downcomer reduces the occurrence and magnitude of sharp gradients and strong mechanical forces, which is beneficial for sensitive organisms like Aurantiochytrium. Additionally, an increase in the Ad/Ar ratio predicts a decrease in the volumetric mass transfer coefficient (kLa), highlighting its importance in controlling the hydrodynamics and mass transfer rates in airlift

systems. It's important to note that mass transfer in the downcomer is approximately 50% lower than in the riser, further emphasizing the need to optimize this ratio to ensure efficient operation and effective oxygen transfer throughout the reactor. With this data, a surface gas velocity of 0.04 m/s was determined. This normalized value is within a range found in the book "Chemical Reactors from Design to Operation" to attribute a homogeneous regime within the reactor. This determined velocity aligns with the established design parameters.

Appendix 13.

Scale Up - Major Equipment

Scale Up - Minor Equipment

Appendix 14.

The equation for Re is (3):

$$Re = \frac{Nd^2\rho}{\mu} \quad (3)$$

ρ = density (kg/m3)

μ =Apparent viscosity of a non-Newtonian fluid (Pa·s)

N = the rotational speed (s −1)

D= Diameter (m) of the nominal rotor (impeller).

Equation (3) describes the geometrical relationship between the rotor diameter and the shear gap distance.

$$R\left(\frac{diameter}{shear\ \ space}\right) = R_{(a)} = \frac{Impeller\ \ diameter\ (D)}{Shear\ \ space\ \ dis\tan ce\ (S)}$$

$$\tau = 0.1894\ln(\gamma) + 2.0303\ \left(R^2 = 0.9772\right) \quad (7)$$

Appendix 15.

	Feed	Liquid	Vapor
Mass Flows (kg/hr)	2179.48	796.906	1382.57
Oil	352.92	352.92	3.8e-06
Ethanol	1164.33	156.652	1007.68
Water	662.23	287.335	374.895

Mole flows (kmol/hr)	62.8922	20.2091	42.6831
Oil	0.8592	0.89592	9.25e-09
Ethanol	25.2736	3.4	21.8732
Water	36.7594	15.9495	20.8098

Appendix 15. Properties of streams in the flash column in HYSYS simulation.

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Appendix 16.

Appendix 16. HACCP analysis and control point system

Process stage	Description of the hazard	Type of Hazard	Preventive measurements
Seed fermentation > Medium sterilization	Medium contamination by bacteria> ***E.coli***	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3)	Revision of the proper functioning of the sterilization equipment: temperature, verification of the sealing of the piping.
	Medium contamination by bacteria> **Aeromonas**	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3).	Revision of the proper functioning of the sterilization equipment: temperature, verification of the sealing of the piping.
	Medium contamination by fungi> **Debaryomyces Hanseni.**	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3).	Revision of the proper functioning of the sterilization equipment: temperature, verification of the sealing of the piping.
	Glucose contamination by > **Aspergillus niger**	Microbiological. Medium severity, low probability of occurrence, Maximum limits (0.00012 CFU/m^3).	Revision of the quality certificate, inspection of the packaging of the input product.
Seed fermentation > Air filtration.	Air contamination by bacteria > ***Actinobacteria> Streptomycetes***	Microbiological. High severity, medium probability of occurrence, maximum	Inspection of equipment operating pressure and membrane integrity.

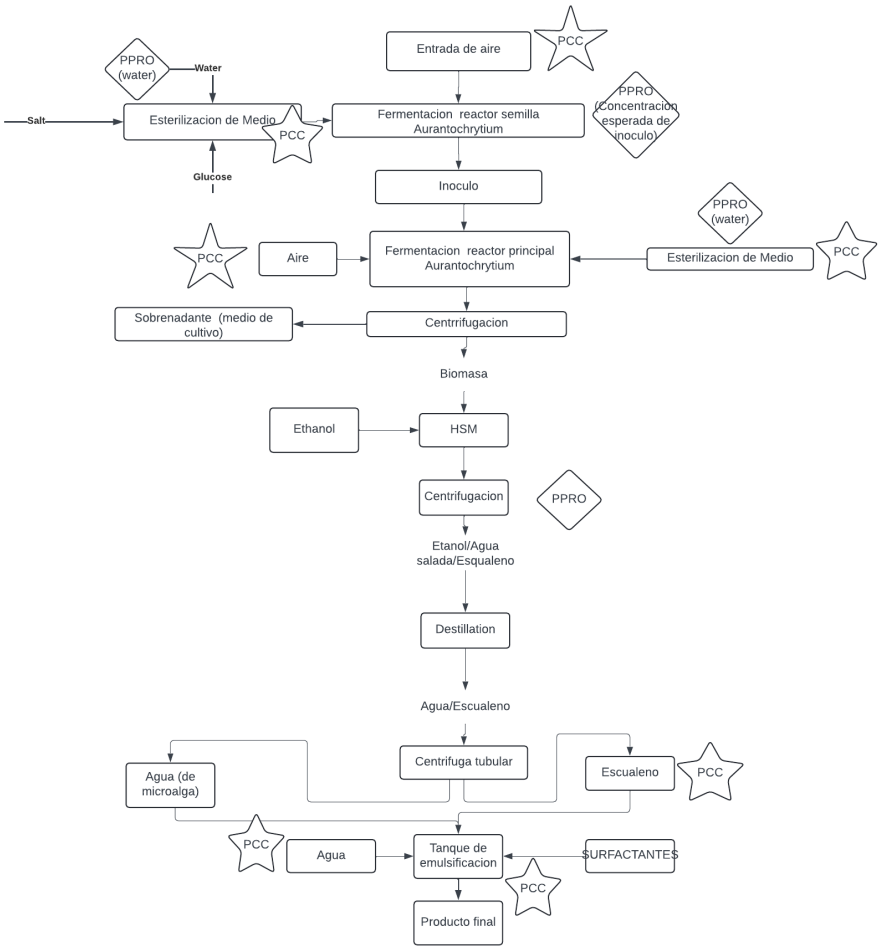
		limits (0.00012 CFU/m^3).	
	Air contamination by bacteria > ***Cladosporium spp.***	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3).	Inspection of equipment operating pressure and membrane integrity.
Medium > Water	Contaminants in water > Residual Chlorine free, solids and pH.	Microbiological. Chemical. Low severity, low probability of occurrence, Maximum Limits (*established by NOM-127-SSA1-1994, pH 6,5-8,5, residual chlorine free 0.2-1.50 mg/L and solid 1g/L	Analysis of ppm, pH and residual chlorine free concentration prior to mixing with glucose and salt.
Air lift fermentation > Medium sterilization	Medium contamination by bacteria> ***E.coli***	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3).	Revision of the proper functioning of the sterilization equipment: temperature, verification of the sealing of the piping.
	Medium contamination by bacteria> **Aeromonas**	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3).	Revision of the proper functioning of the sterilization equipment: temperature, verification of the sealing of the piping.
	Medium contamination by fungi> ***Debaryomyces Hanseni.***	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3)	Revision of the proper functioning of the sterilization equipment: temperature, verification of the sealing of the piping.
	Glucose contamination by > ***Aspergillus niger***	Medium severity, low probability of occurrence, Maximum limits (0.00012 CFU/m^3)	Revision of the quality certificate, inspection of the packaging of the input product.
Air lift fermentation > Air filtration	Air contamination by bacteria >	Microbiological. High severity, medium	Inspection of equipment operating

	Actinobacteria> Streptomycetes	probability of occurrence, maximum limits (0.00012 CFU/m^3).	pressure and membrane integrity.
	Air contamination by bacteria > ***Cladosporium spp.***	Microbiological. High severity, medium probability of occurrence, maximum limits (0.00012 CFU/m^3).	Inspection of equipment operating pressure and membrane integrity.
Medium > Water	Contaminants in water > Residual Chlorine free, solids and pH.	Microbiological. Chemical. Low severity, low probability of occurrence, Maximum Limits (*established by NOM-127-SSA1-1994, pH 6,5-8,5, residual chlorine free 0.2-1.50 mg/L and solid 1g/L	Analysis of ppm, pH and residual chlorine free concentration prior to mixing with glucose and salt.
High shear mixer	Rotor metal particles in the mixture.	Physical. Low severity, low probability of occurrence, Maximum limits (*recommended by FDA, 170 ppm).	Inspection of the physical integrity of the rotor, no tearing of the coating, maintenance of the selected speed.
Lipid extraction	Concentration of squalene in centrifugation supernatant.	Chemical. Medium severity, low probability of occurrence, upper limits (>80 %).	Inspection of the correct functionating of temperature HSM, rotor speed.
Emulsification	Water (current S029) for human use.	Microbiological. Chemical. High severity, probability of occurrence, Maximum Limits (*established by NOM-127-SSA1-1994, pH 6,5-8,5, residual chlorine free 0.2-1.50 mg/L and solid 1g/L).	Inspection that water storage spaces are protected from contamination.
Emulsification	Squalene stability regarding their oxidation.	Chemical. Medium severity, low probability of occurrence, Upper limits (<10 %).	Inspection of sealing of squalene transport pipelines.

Emulsification	Squalene (extract purity)	Chemical. Medium severity, medium probability of occurrence (purity >70%).	Inspection of the adequate functioning of distillation equipment, temperature, pressure.
Emulsification	Squalene (Aztanzontine and Beta caroten concentration as Kg/ Kg of extract)	Chemical. High severity, low probability of occurrence, (Aztanzontine >2 % and Beta caroten > 0.05 %, *As recomended by Tzanova, *et.al.,* 2017)	The correct operation of the centrifuge, through the observation of flow and speed.
Emulsification	Surfactants	Chemical. High severity, low probability of occurrence, Maximum Limits (Purity> 40 %).	Analysis of the manufacturer's quality sheet, physical inspection of the packaging.
Emulsification	Encapsulation efficiency	Chemical. High severity, medium probability of occurrence, Maximum limits (<90).	Inspection of the correct operation of the homogenizing equipment, speed, sealing.

Appendix 17.

Appendix 17.1 Critical Points



Appendix 17.2 Corrective measurements

# PCC	Hazard	Critical Limits	Monitoring			Corrective Measurements	Registration	Responsible for
			What	How	Frequency			
#1 Seed fermentation > Air quality	Air contamination by bacteria	(0.00012 CFU/m^3	The existence of microbial load by *Actinobacteria* and *Cladosporium* spp in the filtered air.	Accordingly, to the United States Environmental protection agency (2014), the sample is taken up SKC	Every 4 hours	The analysis of the HEPA filter membrane integrity. If the quality of the filter is compromised.	The registration of the microbial load as absent (1 CFU/m3) or detected load (CFU/m3).	Technical Quality Analyst

				BioSampler, the resulting liquid is analyzed under microbial growth onto petri dishes.		Discard filter membrane. Repeat the protocol for the use of a new filter, repeat the filtering process.		
#2 Fermentation > Air quality	Filtered Air contamination by bacteria.	(0.00012 CFU/m^3).	The existence of microbial load by Actinobacteria and Cladosporium spp in the filtered air.	Accordingly, to the United States Environmental protection agency (2014), the sample is taken up SKC BioSampler, the resulting liquid is analyzed under microbial growth onto petri dishes.	Every 4 hours	The analysis of the HEPA filter membrane integrity. If the quality of the filter is compromised. Discard filter membrane. Repeat the protocol for the use of a new filter, repeat the filtering process	The registration of the microbial load as absent (1 CFU/m3) or detected load (CFU/m3).	Technical Quality Analyst
#3 Seed Fermentation medium sterilization.	Sterilized medium contaminated by bacteria.	(0.00012 CFU/m^3).	The existence of microbial growth by ***E.coli and* Aeromonas.**	According to NOM-089-SSA1-1994, 1) 100 ml sample is taken, 2) Growth in medium with	Every 9 days.	1)The analysis of the functioning of temperature control on the sterilization process. 2) The analysis of pipe sealing.	The registration of the microbial load as absent (1 CFU/m3) or detected load (CFU/m3).	Technical Quality Analyst.

				standard specifications (Lethen, XLD, dextrose) 3) Determination of microbial growth by observation of turbidity.		3) In case of malfunction of the temperature or sealing, replacement of the system. 4) Esterilization of the medium.		
#4 Fermentation medium sterilization.	Sterilized medium contaminated by bacteria.	(0.00012 CFU/m^3).	The existence of microbial growth by ***E.coli and* Aeromonas.**	According to NOM-089-SSA1-1994, 1) 100 ml sample is taken, 2) Growth in medium with standard specifications (Lethen, XLD, dextrose) 3) Determination of microbial growth by observation of turbidity.	In every charge of medium for the fed batch bioreactor.	1)The analysis of the functioning of temperature control on the sterilization process. 2) The analysis of pipe sealing. 3) In case of malfunction of the temperature or sealing, replacement of the system. 4) Resterilization of the medium	The registration the microbial load as absent (1 CFU/m3) or detected load (CFU/m3).	Technical Quality Analyst.
#5 Squalene (extract purity and antioxi	Squalene concentration on stream #23 and Astanzan	Squalene concentration on lipid oil >70	Kg of squalene (Kgs) per Kg of lipid oil (Kge) and % of Aztanzont	As recommended by cosmetic ingredient review in safety	Every 9 days.	1) Evaluation of the maintenance of mutagenesis in	The registration of the concentration of the extract as Kgs/Kge	Technical Quality Analyst.

dant concentration)	tine and Beta Carotenes concentration.	%. Aztanzontine >2 % and Beta caroten > 0.05 %.	ine and Beta caroten.	assessment of squalene (2019), the analysis of the extract was performed by gas liquid chromatography.		Aurantochrytium. 2) Review of PPRO #1 in order to identify the appropriate cell disruption and squalene concentration. 3) By identifying the main contaminant in the extract, we can A) Perform distillation of the extract again; B) Centrifuge the extract again. 4) If after A and B the concentration is inadequate, the product is discarded.	and %. And Aztanzontine and Beta caroten concentration as %.	
#6 Water use in encapsulation	Water (current S029) for cosmetic use.	Limits established by NOM-127-SSA1-1994, residual chlorine free 0.2-1.50 mg/L and solid 1g/L.	The free residual chlorine and solids in the water.	Chlorine determination with a commercial kit and ppm meter.	Every 9 days.	The water is sterilized through the system used for the fermenter medium.	The registration of Residual chlorine free and total solids.	Technical Quality Analyst.

#7 Encapsulation efficiency.	Emulsification characteristics	200nm< doprelet size> 50 nm. Kg of squalene encapsulated > 90 %.	Droplet size and Kg of squalene encapsulated.	As recommended by International federation of societies of cosmetic chemists (1997) the determination: 1) The analysis of the sample throw a particle size analyzer, 2) The particle size and particle concentration are correlated with the Kg of squalene encapsulated.	Every 9 days	1) An analysis of the pressure, speed and temperature of the homogenizer is performed. 2) If the equipment works correctly, the sample is recirculated for 2 more emulsification cycles. 3) If the sample is still not within the critical limits, it is discarded.	The registration of particle size and % of encapsulated squalene.	Technical Quality Analyst.

Appendix 18

Economic Analaysis

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