Continuous Manoyl Oxide production by a genetically modified strain of *C. reinhardtii*

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***Abstract* — A genetically modified strain of *Chlamydomonas reinhardtii*, tailored for the production and excretion of the terpenoids 13R(+)-Manoyl Oxide (MO) and 9-hydroxy-Manoyl Oxide (9OH-MO), was used in the current work. Both compounds are precursors of forskolin, a potential pharmacy drug. The terpenoids were then extracted by keeping the culture in contact with a dodecane overlayer. Up to 6.7 mg of MO and 1.8 mg of 9OH-MO per gram of dry biomass per day were obtained from cultures growing in batch in Erlenmeyer flasks.**

The aim of this study was to design and test a continuous and scaled up set up for the simultaneous production and extraction of these products. The set up consisted of circulating the microalgae between a flat panel photobioreactor and an extraction vessel, where they would contact the extraction solvent. In 400 mL reactors, 1.8 mg of MO and 0.40 mg of 9OH-MO per gram of dry biomass per day were extracted. Up to 0.76 mg of MO and 0.17 mg of 9OH-MO per gram of dry biomass per day were reached in 1.8 L reactors, where different biomass concentrations and light intensities were tested. A cell death rate between 7 and 9% was observed for the overall process.

The second objective was to further understand the production, excretion and extraction dynamics of the system, to which the remaining terpenoids content inside the cells and in the aqueous phase was assessed. A mass transfer coefficient of $(4.9\pm1.0)10^{-5}$ day^{-1} and $(1.1\pm0.4)10^{-5}$ day^{-1} was calculated for MO in the Erlenmeyer flasks and in the 1.8 L reactors, respectively. In the Erlenmeyers, the production rates were $(6.6\pm0.1)10^{-3}$ $gL^{-1}day^{-1}$ for MO and $(1.7\pm0.1)10^{-3}$ $gL^{-1}day^{-1}$ for 9OH-MO, while $(1.2\pm0.3)10^{-3}$ $gL^{-1}day^{-1}$ and $(2.4\pm1.4)10^{-4}$ $gL^{-1}day^{-1}$ were obtained in the 1.8 L reactors for MO and 9OH-MO, respectively.

Keywords: Chlamydomonas reinhardtii, Terpenoids, Manoyl Oxide, Drug precursor, Continuous extraction, Scale up.

I. INTRODUCTION

Terpenoids are a class of organic compounds commonly found in Nature, with a broad range of applications [1]. Manoyl Oxide is a terpenoid particularly interesting for its potential in the medical field. Both 13R(+)Manoyl Oxide (MO) and its hydroxylated variant, 9-hydroxy-Manoyl Oxide (9OH-MO), are precursors for forskolin, which has shown promising in the pharmaceutical field [2]. Forskolin can be naturally found in the roots of the plant Coleus forskohlii, which has been extensively used in traditional medicine (Coleus). However, plants produce various types of diterpenoids each in small quantities, so if extracted it requires a demanding purification process and large amounts of biomass, leading to low yields. In this way, not only the process is expensive but also not eco-friendly. The existing chemical processes to synthetize this compound are still economically challenging, although recent advancements have been made [4]. Currently the most promising alternatives are in the field of biotechnology. Microalgae have a naturally high turnover of isoprenoids and of the precursor of both pigments and diterpenoids, geranylgeranyl pyrophosphate (GGPP) [1]. For this, microalgae are an option worth exploring.

Chlamydomonas reinhardtii is a green flagellate, first isolated by G. M. Smith in 1945. Its simplicity, rapid growth and high biomass production at low cost (Neupert et al. 2009) made it the most widely studied species in laboratory work, being considered as the type species of the genus Chlamydomonas [6]. Lauersen et al. (2016) has produced genetically modified mutants capable of heterologous expression of terpene synthases, which would not only produce but also excrete the sesquiterpenoid to the surrounding medium. In a similar way, a GMO strain (designated B2) was designed by Lauersen et al. (2018) capable of heterologous expression of diterpene synthases and enzymes from the MEP pathway, leading to the production and excretion of MO and 9OH-MO.

To assure a continuous process, one must extract the compound of interest without compromising the viability of the cells. Hejazi et al. (2002) studied the continuous in situ carotenoids extraction from D. salina, first of their knowledge to extracting carotenoids without intentionally destroying the cells. Kleinegris et al. (2010) built a system where the microalgae were first grown in batch in a flat panel photobioreactor and then stressed to promote carotenoids accumulation. In order to extract the carotenoids, the microalgae were placed in a vessel with a dodecane overlayer, where dodecane was also being sparged from the bottom. The enhanced contact between the cells and the dodecane molecules due to its sparging considerably increased the cell death rate. Additionally, the aeration caused the sparged dodecane to emulsify and spread through the whole vessel [7].

de Boeck (2019) analysed the extraction of MO from B2 strains with different organic solvents, concluding that $\log P_{o/w}$ should be higher than 6, and that dodecane was the best option when considering both the extraction capacity and the cells viability [8].

II. Materials and Methods

A. Strains and cultivation conditions

A genetically modified strain from C. reinhardtii was used (B2). The cultures were inoculated in TAP Medium (Gorman and Levine 1965) and kept in a shake incubator at 25°C with 2% CO2 and 150 µE of light intensity in a 16:8 day-night cycle.

B. Algaebator Experiments

A batch experiment was carried out, where the microalgae were kept in 250 mL Erlenmeyer flasks with 100 mL of culture. The strains were initially kept at 150 µE, which was increased to 318 µE after two days and increased again to 636 µE after one more day. The value 636 µE was chosen since it represents the average Dutch summer light in block [10], and 318 µE for being half of it. The mixture of the culture was assured by using magnetic stirrers. After one day in the incubator, a 10 mL dodecane overlayer was added to the flasks.

C. Infor reactors Experiments

Four Infor reactors were inoculated with T2P 2N Medium (TAP Medium with HCl instead of acetic acid and twice the phosphorus and nitrogen concentration), a pH set point at 7.2 and base addition to control it, with an airflow of 1L. Two of these reactors (A and B) were grown in batch until the desired biomass concentration was reached, during which the primary light was manually gradually increased. The other two were grown under luminostat with 50 µE of outgoing light. The reactors were then run under turbidostat mode during the extraction, to assure a steady state.

D. Extraction system

The reactor would be connected to an extraction vessel, so that the microalgae would be constantly circulating from the reactor into the extraction vessel and back to the reactor again (Fig. 1). A pump was needed to assure the flow and control its speed, both in and out the extraction vessel. Silicone tubes with 1.5 mm of internal diameter would transport the culture between the two vessels, and Watson-Marlow Marprene tubes with 1.42 mm internal diameter were used for the pump. A Watson-Marlow multi-channel peristaltic pump was used, model 205 U, at 90 rpm. Each path used two of the pump's channels working in parallel.

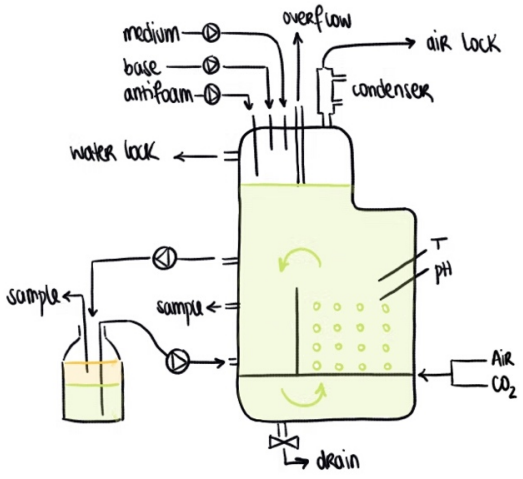

Figure 1. Schematic of the designed set-up applied for the Infor reactors.

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E. GC Analysis

The terpenoids concentration in the dodecane was determined using on a GC-FID 7890A from Agilent using a RESTEK Rxi-5ms column 0.25 µm (5% diphenyl, 95% dimethylpolysiloxane).

III. Results and Discussion

A. Reactor Process Design

Some changes had to be made from the first design to the one used in the experiment, mainly to increase the flow inside the tubes carrying the algae in and out the extraction vessel. Initially the liquid had a flow rate of 5.56×10^{-8} $m^3 s^{-1}$, which corresponded to a speed of 4.42×10^{-3} ms^{-1} and a Reynolds number of 17.7. At such a low speed, the biomass was depositing inside the tubes at a rate higher than expected for the wild type due to the reduced mobility from these strains, a consequence from having no flagella, compromising the whole process. In order to solve this problem, thinner tubing was used, as well as two pumps in parallel for each way. This increased the flow rate to 2.55×10^{-7} $m^3 s^{-1}$, the speed to 0.144 ms^{-1} and Reynolds to 216.

The cells viability was assessed both right before the extraction system was turned on and at the end of the experiment, in order to evaluate its effect on the cells. The percentage of non-viable cells increased from 2-4% to 7-9% during the time the cells where submitted to the pump and contact with dodecane. These values are lower than the ones registered for Dunaliella Salina in contact with a dodecane overlayer, where a cell death rate of around 20% was observed after four days [7].

B. Biomass and terpenoid productivities

In the Algaebator, microalgae were growing with a biomass productivity of $(3.7 \pm 1.5) \cdot 10^{-1}$ $gL^{-1}day^{-1}$. These microalgae were responsible for 3.2±4.7 $mgg^{-1}day^{-1}$ of MO and 1.0±2.7 $mgg^{-1}day^{-1}$ of 9OH-MO. Four Infor reactors were running in turbidostat at different biomass concentrations, with the designed extraction set up. Their productivities are summarized in table 1. From comparing the three runs at 636 µE, we can observe that the extracted MO per biomass remains similar in all of them, while the productivities per volume of culture and light both increase with the increase of biomass concentration, as expected. Looking at reactors B and C, which have a considerable similar biomass concentration, we can see that the biomass productivities are almost the double at the highest light intensity, while the light productivity is understandably lower. Similar trends can be seen for the 9OH-MO.

All productivities are considerably lower than the ones registered in the Algaebator experiment. The main reasons for this are a lower area to volume ratio and a smaller proportion between the time spent in contact with the extraction solvent and the time spent inside the reactor.

TABLE I. OPTICAL DENSITIES (OD), BIOMASS CONCENTRATION (CM), MANOYL OXIDE (MO) AND 9-HYDROXY-MANOYL OXIDE (9OH-MO) PRODUCTIVITIES AND PRIMARY LIGHT INTENSITIES FOR THE FOUR STUDIED SCENARIOS.

Infor	OD (750 nm)	x (g/L)	MO·10¹ ($mgL^{-1}day^{-1}$)	MO·10¹ ($mgg^{-1}day^{-1}$)	MO·10¹ (mg/mol_{light})	Light (µE)
A	3.0±0.7	1.4±0.3	5.8±4.8	4.5±3.6	2.1±1.8	636
B	5.1±0.7	2.3±0.3	8.3±14.5	3.7±6.7	3.0±5.3	636
C	4.3±0.5	2.0±0.2	15.5±6.5	7.6±2.6	1.9±0.8	1877
D	1.7±0.2	0.79±0.09	4.2±3.3	5.3±3.9	1.5±1.2	636
Infor	**OD (750 nm)**	**Biomass ($gL^{-1}day^{-1}$)**	**9OH-MO·10² ($mgL^{-1}day^{-1}$)**	**9OH-MO·10² ($mgg^{-1}day^{-1}$)**	**9OH-MO·10² (mg/mol_{light})**	**Light (µE)**
A	3.0±0.7	0.86	21±23	17±17	7.8±8.3	636
B	5.1±0.7	0.11	18±33	8±16	6±12	636
C	4.3±0.5	0.044	26±18	13±11	3.4±2.6	1877
D	1.7±0.2	0.20	7.4±6.5	9.5±8.5	2.7±2.4	636

C. Manoyl Oxide production and transfer mechanism

This section is dedicated to the understanding of the production, excretion from the cells and transfer from aqueous to organic phase mechanisms. This study was made based on the model created by Heinrich et al. (to be published), first created assuming steady state, such as the one reached when the reactors are operating in turbidostat mode. Further on, it was adapted for a transitory state, to describe the situation from the algae growing inside the Erlenmeyers in the algaebator. Based on mass balances to the biomass and to the MO inside the cells, in the dodecane layer and in the aqueous phase, the following differential Equation System was reached:

$$\mu = \frac{Q}{V_R} \tag{1}$$

$$\frac{dm_{MO}^{X}}{dt} = \frac{r_{MO}}{x} - \frac{r_{EX}}{x} \tag{2}$$

$$\frac{dm_{MO}^{DD}}{dt} = \frac{V_R}{V_{DD}} k_T a\left(K_4 m_{MO}^{AQ} - m_{MO}^{DD}\right) \tag{3}$$

$$\frac{dm_{MO}^{AQ}}{dt} = -\frac{Q}{V_R}\left(m_{MO}^{AQ} + x m_{MO}^{X}\right) - k_T a\left(K_4 m_{MO}^{AQ} - m_{MO}^{DD}\right) + r_{EX} \tag{4}$$

The terpenoids concentration in the aqueous phase and inside the cells was seen to be constant over time. Knowing that K_4 has the value of $10^{5.5}$ for MO $10^{4.57}$ to 9OH-MO (Chemicalize), the mass transfer coefficients (k_Ta) and production and excretion rates (r_{MO} and r_{EX}) were calculated. For r_{MO}, the value of $(1.2\pm0.3)10^{-3}$ $gL^{-1}day^{-1}$ was obtained for MO and $(2.4\pm1.4)10^{-4}$ $gL^{-1}day^{-1}$ for 9OH-MO. The k_Ta was $(1.1\pm0.4)10^{-5}$ day^{-1} for MO and (7.8 ± 2.6) 10^{-5} day^{-1} for 9OH-MO. The mass transfer coefficient is almost one order of greatness bigger for the 9OH-MO than for the MO, since the decrease in K_4 increases k_Ta, and the concentration of 9OH-MO in the aqueous phase is lower than for MO. Using average concentration values and the specific growth rate, the fractions of MO productivity in the pellet, supernatant and dodecane overlayer where calculated, as can be seen in the next graphs. Looking at the three different biomass concentrations subjected to a light intensity of 636 µE in bottom graph (Fig. 2), it is observable that the higher the biomass concentration, the less MO per gram of biomass is produced. When comparing the two bars on the right side of the graph, which have a quite similar biomass concentration but different ongoing light values, the productivity in the dodecane from the reactor with 1877 µE of primary light is almost the double from the one at 636 µE.

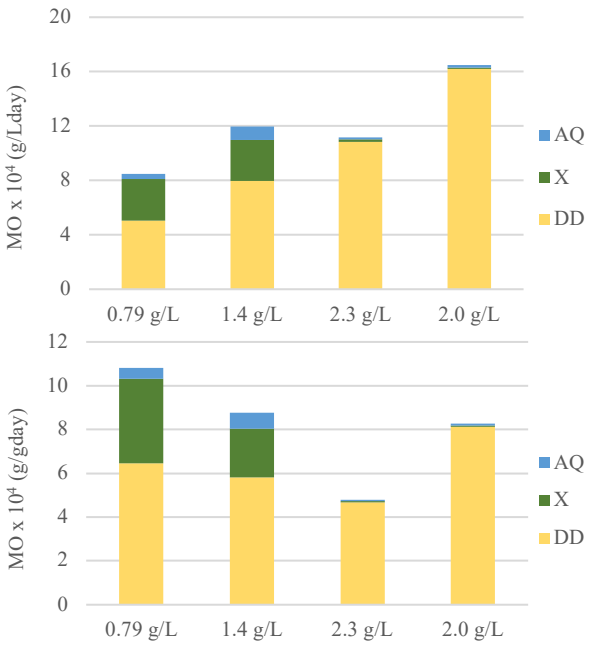

Figure 2. MO productivity fractions in the dodecane layer (DD), pellet (X) and aqueous phase (AQ) in g per L of culture per day (top) and in g per g of culture per day (bottom). The first three runs (0.79, 1.4 and 2.3 g/L) had a light intensity of 636 µE, while the last one (2.0 g/L) was at 1877 µE.

Since a lower biomass concentration leads also to a higher light per cell, this seems to be an important parameter for the production of MO. Further on, we can see two different paths towards increasing this parameter, keeping a lower biomass concentration or a higher primary light. From the top graph we get an indication to go through the second one, given that volume is an actual limitative logistical constrain to bear in mind. One must also consider the available sun irradiance from the place where the scaled-up project would be, in another words, if the natural conditions of the location allow for such a choice.

Our hypothesis was that the concentration of MO in the aqueous phase would affect the efficiency of the process in two opposite ways. Due to an inhibition effect, an increase in concentration would decrease the production efficiency. On the other hand, it would promote the mass transfer from the aqueous to the organic phase, increasing the extraction efficiency. To assess this, the fractions of MO productivity in the dodecane and in the pellet were plotted alongside the MO concentration in the aqueous phase. The experimental data indicate that more MO is extracted with the increase in MO concentration in the aqueous phase, whilst the MO inside the pellet decreases with that same concentration. This corroborates the mentioned hypothesis and indicates not only that the mass transfer of the terpenoid from the cell to the organic solvent occurs through the aqueous phase, but also that the terpenoid concentration in the medium is a key parameter for the efficiency of the process. This is, however, a parameter harder to tune than the ingoing light, given its contradictory effects in the system. Nevertheless, a higher MO concentration in the aqueous phase, and therefore a higher biomass concentration, seems to be a better option given that the amount of product which we get matters more than the one we are able to produce but not to recover.

The trend is however significantly different if the same analysis is made looking at the 9OH-MO. For this terpenoid, an ideal range of biomass concentration for the tested conditions seems to have been found, as not only the total productivity but also all its fractions (in the aqueous phase, in the pellet and in the dodecane) show a maximum value for the reactor with 1.4 g/L of biomass.

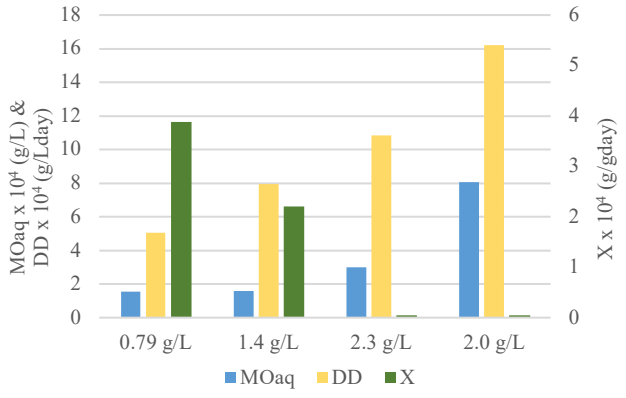

Figure 3. MO concentration in the aqueous phase in g/L (MOaq) and MO productivity fractions in the dodecane (DD), in g per L of culture per day, and in the pellet (X), in g per g of culture per day. The first three runs (0.79, 1.4 and 2.3 g/L) had a light intensity of 636 µE, while the last one (2.0 g/L) was at 1877 µE.

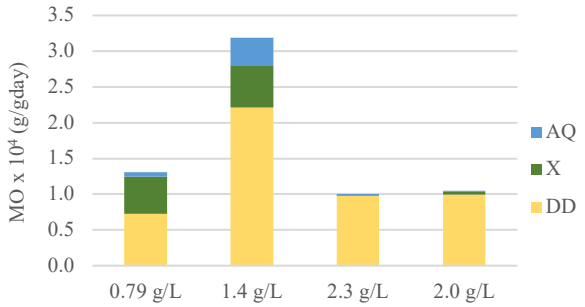

Figure 4. 9OH-MO productivities in the dodecane layer (DD), pellet (X) and aqueous phase (AQ) in g per g of culture per day. The first three runs (0.79, 1.4 and 2.3 g/L) were with 636 µE, while the one at 2.0 g/L was at 1877 µE.

There is no clear relation between the fraction of productivity in the pellet and the terpenoid concentration in the medium, which indicates the its presence does not inhibit the cell from converting more MO into 9OH-MO. The lack of relation between the extracted 9OH-MO and its concentration in the medium is more intriguing. When looking only at the reactors operating at 636 µE, it can still be seen that the more 9OH-MO present in the medium, the more it is extracted, as expected. However, the reactor at 1877 µE exhibits the highest product concentration in the medium and the second lowest productivity fraction in the dodecane. This could also be the result of an error in the GC-FID analysis, given that this quantitative analysis tends to be less exact for smaller concentrations.

When plotting the total 9OH-MO productivity alongside with the specific growth rate (Fig. 6), it can be seen that both these variables evolve in the same way. This highlights the close link between growth and terpenoid production from the cells, and how optimizing the conditions for one will probably optimize them for the other as well. If one would assure a higher growth rate even at higher biomass concentrations, by, for example, supplying a richer medium to the cells, the MO production would probably increase. Given that the medium had twice the nitrogen and phosphate concentrations than the original recipe, which should allow the microalgae to grow to higher optical densities than the ones observed, the trace elements influence should be further analysed and eventually increased in order not to limit the cells growth at higher biomass concentrations. On the other hand, a higher dilution rate implies a higher loss of product with the overflow. This is however, directly connected to the used set up, and a concern which would not exist in different and scaled up reactor designs.

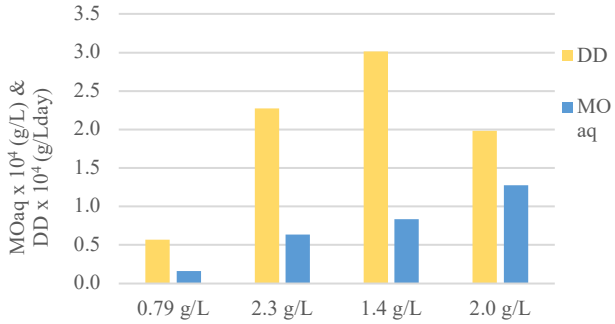

Figure 5. 9OH-MO productivity fraction in the dodecane in g per L of culture per day (DD) and MO concentration in the aqueous phase in g/L (MOaq). The three runs were with 636 µE, the other was at 1877 µE.

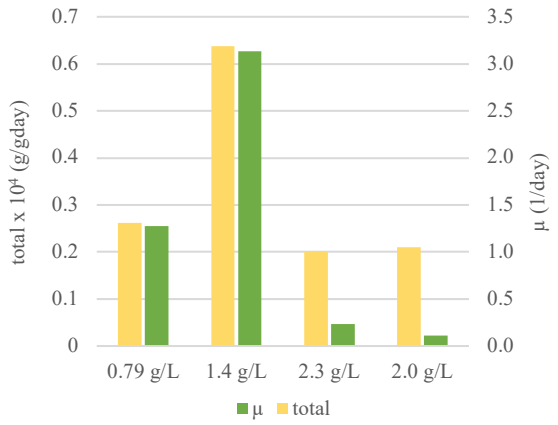

Figure 6. 9OH-MO total productivity per mass of culture and specific growth rate (μ). The first three runs (0.79, 2.3 and 1.4 g/L) had a light intensity of 636 μE, while the last one (2.0 g/L) was at 1877 μE.

To conclude, in order to enhance MO and 9OH-MO production and excretion from the B2 strain and their transfer to the extraction solvent, one must optimize the growing conditions for this strain and improve the extraction system design by increasing the mass transfer area per volume ratio and avoiding the loss of product with discarded culture.

The model by Heinrich *et al.* (to be published) was also applied to the experiment in the algaebator, where the system was in transitory state. There are two main differences to take into consideration: first there is biomass growth, second there is no medium input nor overflow. For these reasons, equations 1 and 4 from the differential equation system where substituted by 5 and 6, respectively.

$$\frac{dx}{xt} = r_x \tag{5}$$

$$\frac{dm_{MO}^{AQ}}{dt} = -k_T a\left(K_4 m_{MO}^{AQ} - m_{MO}^{DD}\right) - m_{MO}^{X} r_X + r_{EX} \tag{6}$$

Once again, the MO content in the aqueous phase and pellet where plotted, and once again those concentrations remained roughly constant over time. The k_Ta was calculated through equation 3, and then used to calculate the production rate, obtaining the values of $(4.9\pm1.0)\cdot10^{-5}$ day^{-1} and $(6.6\pm0.1)\cdot10^{-3}$ $gL^{-1}day^{-1}$, respectively. Regarding the 9OH-MO, a k_Ta of $(3.7\pm1.2)\cdot10^{-4}$ day^{-1} and a production rate of $(1.7\pm0.1)\cdot10^{-3}$ $gL^{-1}day^{-1}$ were obtained. Once again, the mass transfer coefficient was higher for 9OH-MO than for MO. The proportion between the produced MO and 9OH-MO sustains what was previously observed. The production rate is around 6 times higher in the *Algaebator* than in *Infor* reactors, which is consistent with the previous results, due to a more efficient and constantly on-going extraction.

IV. Conclusion

The productivities obtained for the cultures growing in batch in Erlenmeyer flasks, up to 6.7 mg of Manoyl Oxide and 1.8 mg of 9-hydroxy-Manoyl Oxide per gram of dry biomass per day, were lower than the ones recorded in literature, which could be due to an erroneous quantitative analysis.

It was possible to continuously extract both terpenoids in a circulating system, even though a smaller mass transfer area to volume ratio and a lower contact time between the cells and the extraction solvent led to smaller titres. Something to bear in mind while building such a set-up, is to assure a culture flow fast enough to prevent biomass deposits.

Optimizing the growth conditions, such as biomass concentration, incident light intensity and medium composition, would improve the production rate. For Manoyl Oxide, a higher biomass concentration and primary light increase its production, while a maximum for 9-hydroxy-Manoyl Oxide production seems to have been found for a biomass concentration around 1.4 g/L at 636 μE of light intensity.

This continuous extraction system was developed for Manoyl Oxide and its hydroxylated variant, but it could be easily applied to any other *C. reinhardtii* strain producing any other terpenoid.

Acknowledgement

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References

[1] K. J. Lauersen *et al.*, "Phototrophic production of heterologous diterpenoids and a hydroxy-functionalized derivative from Chlamydomonas reinhardtii," *Metab. Eng.*, vol. 49, no. July, pp. 116–127, 2018.

[2] S. Khatun, "The Strategies for Production of Forskolin vis-a-vis Protection Against Soil Borne Diseases of the Potential Herb Coleus forskohlii Briq.," *European J. Med. Plants*, vol. 1, no. 1, pp. 1–9, 2011.

[3] Coleus, "Coleus." [Online]. Available: https://www.webmd.com/vitamins/ai/ingredientmono-1044/coleus. [Accessed: 06-Mar-2019].

[4] I. Pateraki *et al.*, "Total biosynthesis of the cyclic AMP booster forskolin from Coleus forskohlii," *Elife*, vol. 6, pp. 1–28, 2017.

[5] J. Neupert, D. Karcher, and R. Bock, "Generation of Chlamydomonas strains that efficiently express nuclear transgenes," *Plant J.*, vol. 57, no. 6, pp. 1140–1150, 2009.

[6] E. H. Harris, "Chlamydomonas in the Laboratory," *Chlamydomonas Sourceb.*, pp. 241–302, 1989.

[7] D. Kleinegris, *Milking of Microalgae revisited*, vol. 22. 2010.

[8] R. De Boeck, "Designing an in-situ extraction of manoyl oxide produced by Chlamydomonas reinhardtii," Wageningen University & Research, 2019.

[9] D. S. Gorman and R. P. Levine, "Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 54, no. 6, pp. 1665–1669, 1965.

[10] G. Benvenuti, "Batch and repeated-batch oil production by microalgae," Wageningen University & Research, 2016.

[11] Chemicalize, "Chemicalize." [Online]. Available: https://chemicalize.com. [Accessed: 11-Oct-2019].