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Production of Omega-3 Oil by *Aurantiochytrium mangrovei* Using Spent Osmotic Solution from Candied Fruit Industry as Sole Organic Carbon Source

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Abstract: Osmotic dehydration is an important phase in the production of dried products, including most fruits and vegetables, in the food industry. The drying process for candied fruit produces a liquid waste called "spent osmotic solution", which is characterized by a high content of organic compounds, mostly dissolved sugars. The sugar content of this food by-product could be valorized through the growth of biomass with a high added value. In this study, the spent osmotic solution from the candied fruit industry was used as an organic carbon source for the growth and production of docosahexaenoic acid (DHA) in the cultivation of *Aurantiochytrium mangrovei* RCC893. The carbon content of the standard media was completely replaced by the sugars present in this food by-product. After that, the growth condition of this strain was optimized through response surface methodologies using a central composite design (CCD), and the optimal combination of the spent osmotic solution and nitrogen was established. Moreover, a scale-up trial was performed using the optimal conditions obtained after CCD to evaluate the scalability of the process.

Keywords: sustainability; PUFA; food waste; DHA; microalgae

1. Introduction

Omega-3 (ω-3) and omega-6 (ω-6) long-chain polyunsaturated fatty acids (LC-PUFAs) are compounds that have long been studied and discussed. In particular, docosahexaenoic acid (DHA, C22:6n-3) has been widely studied because it is an important fatty acid for human health with a series of benefits. It is the most abundant LC-PUFA in the human brain and one of the major components of the central nervous system, essential for brain growth and development in infants. [1]. For that reason, DHA is used in many adult supplements and infant formulas [2].

The principal source of DHA is fish oil obtained from fatty fish (i.e., mackerel, salmon and tuna), but it has several disadvantages, including the low sustainability for the overexploitation of marine biotic resources; contaminations by marine pollutant are characterized by an undesirable fishy smell [3,4]. All these factors have caused concern over the long-term sustainability and safety of fish oil, increasing the attention on new sources of LU-PUFAs.

Single-cell organisms, especially microalgae, received great interest from the scientific community due to their capacity to accumulate large amounts of LC-PUFAs in a controlled environment.

Thraustochytrids are heterotrophic protists commonly found in marine environments and are widely known for their high concentration of ω3 LC-PUFA, especially DHA [5]. Omega-3 oil obtained from thraustochytrids is a potential alternative to fish oil because

of its high biomass productivity and DHA content, which is much higher than that of the fish source [6]. Among the thraustochytrids, *Aurantiochytrium* (known as *Schizochytrium* until 2007), a protist commonly found in many coastal ecosystems, is one of the highest LC-PUFAs producers [7]. It can produce a large amount of lipids (up to 60% of dry weight), most of which is DHA (up to 55% of total fatty acids) [7,8]. This protist is commercially used in DHA-rich oils and as a food ingredient in foods, feeds and nutritional supplements [5], and it is free from common algal toxins (i.e., domoic acid) [9]. Industrial production of DHA by thraustochytrids requires a great amount of glucose and yeast extract (as a nitrogen source), which makes the process expensive. In fact, the nutrient source represents a significant portion of the production costs for heterotrophic cultivation [10].

To overcome this issue, many efforts have been carried out to research new sustainable nutrient sources for microalgae cultivation. One of the most promising alternatives to standard nutrients is the utilization of food by-products as a medium for the growth of algae biomass [11].

In fact, the use of sugar-rich by-products derived from the agro-food industry could represent a sustainable alternative to reduce omega-3 oil production costs and concurrently valorize food waste.

Spent Osmotic Solution (SOS) from the candied fruit industry is an interesting by-product that could be used for the cultivation of heterotrophic aquatic protists. This waste is generated after the osmotic dehydration of fruits (cherry, orange, berries, etc.) in order to preserve the aroma, extend shelf life and reinforce the sweet taste of fruits [12]. The disposal of this food waste has a high economic impact and represents an environmental problem due to the high chemical oxygen demand (COD) and low pH. Few attempts have been carried out to recycle or valorize this industrial waste, and the most promising approach is the biotechnological conversion. Aachary and Prapulla (2009) successfully converted SOS into fructooligosaccharides (FOS) through the transfructosylation reaction of the fructosyl transferase enzyme produced by *Aspergillus oryzae* MTCC 5154 [12]. However, SOS has never been tested as a growth medium for aquatic protists.

Recently, the extremophile red algae *Galdieria sulphuraria* was grown using a similar spent brine liquid, resulting in a change in the algal biochemical composition and valorizing the food waste [13]. Moreover, *Aurantiochytrium* sp. have been successfully grown on other food by-products thanks to their metabolic feasibility [11,14,15], but the strain RCC893 has never been tested on any type of food processing by-product.

Therefore, the purpose of this study was to investigate the potential of spent osmotic solutions from the candied fruit industry as a carbon source for the cultivation of *Aurantiochytrium mangrovei* RCC893. The growth factors were optimized through response surface methodologies, and a scale-up trial was also performed. We proved that it is possible to use this food by-product as a low-cost carbon source for the production of biomass rich in lipids and DHA.

2. Materials and Methods

2.1. Organism and Cultivation

A. mangrovei (RCC893) was obtained from the Roscoff algae collection (France). A stock culture of an axenic microalga strain was maintained routinely by regular sub-culturing at 2-week intervals on both liquid and agar slants of YEP medium following the recipe provided by the Roscoff collection. YEP broth was obtained from filtered natural oligotrophic seawater adjusted at pH 6.5. The nitrogen (N) sources were peptone (2 g L^{-1}) and yeast extract (2 g L^{-1}), while the organic carbon source was glucose in a concentration of 20 g L^{-1}. As a microelements supplement, 1 mL L^{-1} of metal solution was added to the media, consisting of: $MgSO_4 \cdot 7H_2O$ (200 mg L^{-1}), KH_2PO_4 (200 mg L^{-1}), $NaHCO_3$ (100 mg L^{-1}), $MnCl_2 \cdot 4H_2O$ (9 mg L^{-1}), $Fe_3Cl_3 \cdot 6H_2O$ (3 mg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (1 mg L^{-1}), $CoSO_4 \cdot 5H_2O$ (0.3 mg L^{-1}) and $CuSO_4 \cdot 5H_2O$ (0.2 mg L^{-1}), as well as 0.1 mg L^{-1} of thiamine [16].

The microalga was cultivated in dark conditions at a temperature of $24 \pm 2\,°C$. Culture agitation was provided by means of an orbital shaker at 140 rpm.

2.2. Spent Osmotic Solution Samples

SOS samples were provided by a local factory in Naples, Italy that produces candied fruits. The samples were frozen at $-24°C$ to prevent any kind of fermentation.

In prior chemical analyses, SOS samples were centrifuged at $5000\times g$ for 15 min at $10\,°C$, and then the supernatant was collected and the solid fraction discarded.

The chemical composition of SOS was: dry weight (DW) 702.11 g Kg^{-1}; total nitrogen (TN) 0.012 g Kg^{-1}; total sugars 682.23 g Kg^{-1}; reducing sugars 279.72 g Kg^{-1}; ash content 0.25 g Kg^{-1}; and pH 5–5.3.

2.3. Experimental Design

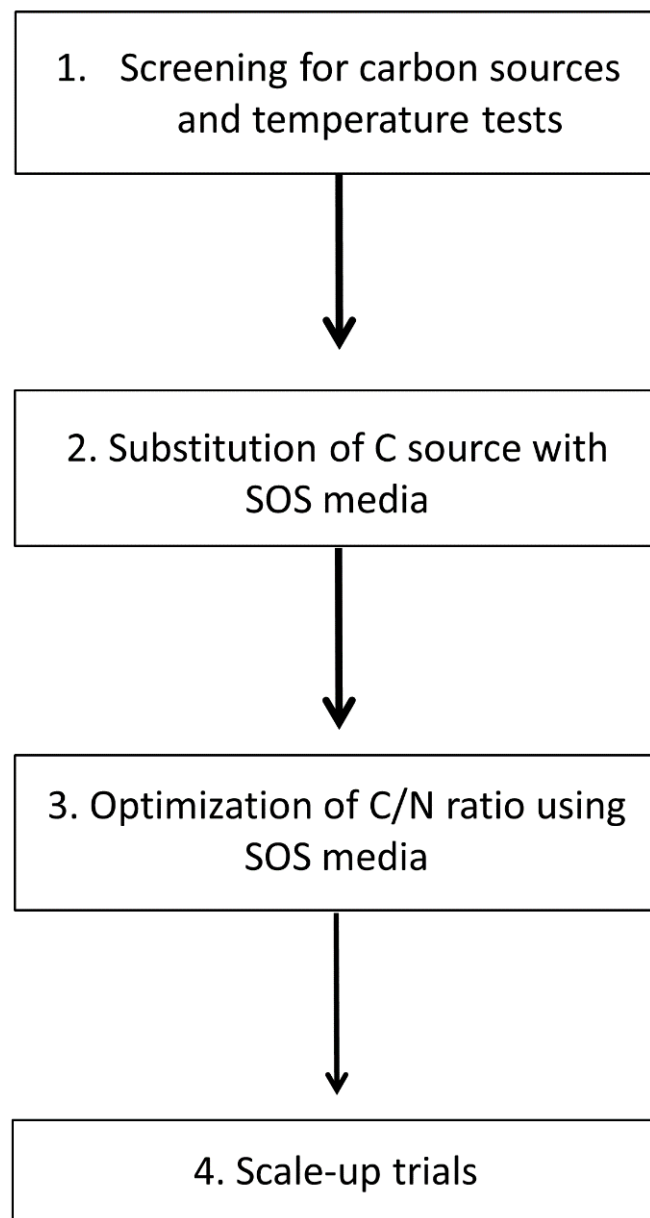The experimental design is summarized in Figure 1.

Figure 1. Scheme of experimental design proposed in this study.

It was divided into four parts:

(1) Screening test to evaluate the growth of *A. mangrovei* RCC893 using different temperatures and different organic carbon sources in order to define the growth performances and the best operating parameters;
(2) Substitution of C source in the standard media using SOS as an organic carbon source;
(3) Optimization of biomass and DHA production through response surface methodologies (RSM) with different C/N ratios;
(4) Scale-up trial in airlift reactor to evaluate the scalability of the new SOS medium.

2.3.1. Best Growth Temperature and Organic Carbon Source

For the determination of the optimal temperature conditions, five different temperatures were tested (20, 24, 28, 32 and 36 °C) by means of a shaking thermostatic bath. Three Erlenmeyer flasks for each temperature were prepared with a standard medium and a working volume of 100 mL with a rotary speed of 140 rpm. The inoculum level of *A. mangrovei* was set at 10% v/v for all the tested temperatures.

For the evaluation of the organic carbon source, four different types of organic carbon were tested (glucose, fructose, sucrose and glycerol) by adding 20 g L^{-1} of each to YEP broth without any other source of organic carbon. Each run was performed in triplicate. For this experiment, a working volume of 120 mL was placed in a 250 mL Erlenmeyer flask and *A. mangrovei* was inoculated into each flask to reach an initial DW of 400 mg L^{-1}. The dry cell weight was evaluated every 24 h.

2.3.2. Substitution of Carbon Source with Spent Osmotic Solution

To evaluate the potential of SOS, the sugars present in this food waste were used to replace the glucose in the standard media at different percentages of substitution. To achieve that, the carbon content provided by glucose in YEP broth was replaced at 25, 50, 75 and 100% by sugars present in SOS. That means that at 100% substitution, no glucose was added to the media. The differences between the standard media and the one obtained with the food by-product were analyzed through ANOVA.

The trial was conducted on an orbital shaker at 140 RPM at 28 °C in triplicate, and the inoculum level of *A. mangrovei* was set at 10% v/v.

2.3.3. Response Surface Analysis and Formulation of Optimized Media

The response surface method (RSM) was applied to determine the optimal combination of SOS (g L^{-1}) and yeast extract (nitrogen source) by constructing a three-level full factorial, central composite design (CCD). The optimization consisted of 14 runs conducted in 2 blocks with 4 cubic points (or factorial points), 4 axial points (or star points) and 3 center points for each block.

The mathematical relationship of the response (Y) to the significant independent variables X1 and X2 is given by the following quadratic polynomial Equation (1):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \beta_{ij} X_i X_j \tag{1}$$

where Y is the predicted response; X_i and X_j are the coded values; β_0 the independent coefficient; $\beta_{i,j}$ is the linear coefficient associated with each independent factor ($X_{i,j}$); and β_{ij} and β_{ii} are the coefficients for interaction and quadratic effects, respectively [9].

The factors selected for this test were yeast extract (YE) and sugars from SOS, both expressed in g L^{-1}.

Two responses were taken for examination for this study: biomass productivity (expressed in g/L/day) and DHA productivity (expressed as mg/L/day).

A duplicate for each run was prepared in an air-lift bioreactor with a working volume of 300 mL. Culture mixing was provided by means of an air bubbling system equipped with a filter of 0.22 μm to avoid culture contamination.

Finally, the optimized medium was used in a scale-up trial with an air-lift bioreactor of 5 L. The culture oxygenation and mixing were provided through an air bubbling system (flow rate 2.5 L min^{-1}) equipped with a filter of 0.22 μm, and the temperature was maintained at 28 ± 1 °C. Growth was carried out in the dark.

2.4. Analytical Methods

2.4.1. Measurement of Dry Cell Weight and Residual Nutrients

For all the growth tests, aliquots of the culture volume were taken every 24 h and transferred in weighted dry tubes, then centrifuged at 5000× *g* for 10 min. The supernatants were discarded and the pellet was washed with phosphate buffered saline (PBS) and dried overnight in the oven at 105 °C to obtain the dry cell weight (DCW) [14].

The biomass productivity (g/L/day) was calculated using the following equation:

$$\text{Productivity} = (\text{final DCW content} - \text{initial DCW content})/\text{cultivation time}$$

For the determination of residual sugars, samples were withdrawn from flasks and collected in sterile tubes and filtered prior to analysis. The sugar content during cultivation was determined using the Dubois method assay [17].

2.4.2. Fatty Acid Methyl Esters (FAMEs)

Samples were analyzed by GC-MS (Agilent GC7890A-MSD5975C) coupled by an Elementar GC5 combustion oven to an IRMS (Elementar Isoprime 100).

In CSIA (Compound-Specific Isotope Analysis) mode, samples were prepared according to adapted Bligh and Dyer (1959) and Morrison and Smith (1964) protocols: liquid–liquid lipid extraction from 50 mg samples with a chloroform–methanol–water mixture (2-2-1.8 ratios) with ball mill grinding [18,19]; and hydrolysis and fatty acid extraction and transesterification at 100 °C for 60 min with toluene and 7% boron trifluoride diluted in MeOH (1-1 volume). FAMEs were identified by chromatographic comparison with authentic standards (Sigma Chemical Co., St. Louis, MO, USA). The quantity of DHA was estimated from the peak areas on the chromatogram using nonadecanoic acid (19:0) as an internal standard.

2.5. Statistical Analysis

All the analyses were carried out in triplicate, and average values with standard deviation were reported. One-way ANOVA was applied using raw data to test for significant differences among the samples (the significance level was always set at $p < 0.05$). The Tukey's test was used as post hoc analysis when there were significant differences among the samples. The data were analyzed using SPSS Statistics software Ver. 23 (SPSS, Inc., Chicago, IL, 2020). The optimization process was evaluated with an RSM analysis, performed in 'R' (RStudio with 'R' version 3.0.2, RCore Team, Vienna, Austria, 2020).

3. Results and Discussions

3.1. Optimal Standard Conditions

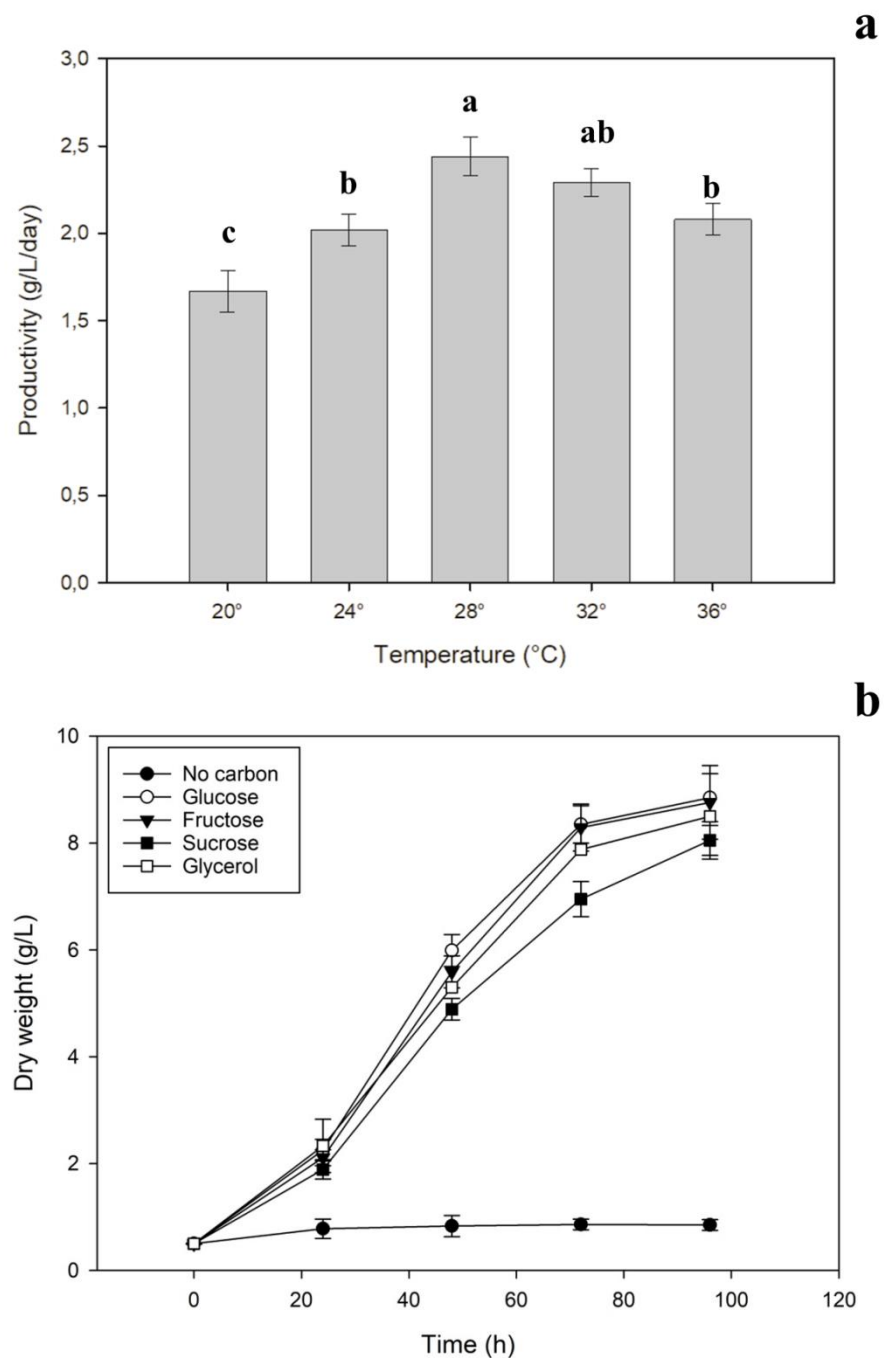Figure 2a reports the biomass productivity of *A. mangrovei* RCC893 at different temperatures.

Figure 2. Productivity and growth curves of *A. mangrovei* RCC893 at different growth temperatures (**a**) and organic carbon source (**b**) on an orbital shaker. Values are reported as mean ± SD. Different letters in the same graph show the statistical difference among the treatments.

At 20 °C, the microalga registered the lowest productivity, while the highest biomass production was obtained at 28 °C. Temperatures higher than 32 °C resulted in a lower biomass productivity compared to the one growth at 28 °C. Based on these results, all subsequent tests were conducted at 28 °C.

This result is in line with a study by Nakazawa et al. (2012) that evaluated the growth behavior of *Aurantiochytrium* sp. strain 18W-13a at 10–35 °C, as well as a study by Taoka et al. (2009), which used strain mh0186 of *A. limacinum* [20,21].

The evaluation of different carbon sources on *A. mangrovei* RCC893 growth is reported in Figure 2b. No significant differences were observed between glucose, fructose and glycerol, which were the best carbon sources with the highest final concentrations (between

8.5–8.8 g L^{-1}). Media supplemented with sucrose reported a lower productivity after 72 h of cultivation. However, after 96 h of cultivation, productivity was not statistically different to that of the other sugars. Different works reported a similar screening for different C sources on *Aurantiochytrium* sp. growth. Yu et al. (2015a) reported a significant growth for *Aurantiochytrium* sp. YLH70 when cultivated with sucrose, but with a growth performance lower than that of glucose and fructose [15]. This result is in line with our study. Mariam et al. (2021) reported glycerol, glucose and fructose as the best C sources for a tested indigenous thraustochytrid. However, the authors showed that this strain was unable to metabolize sucrose [22]. Moreover, Pahlavanyali et al. (2020) reported that hydrolysis of sucrose in a molasses-based medium was necessary in order to improve biomass and DHA production from *Schizochytrium* sp., remarking that sucrose, as a main organic carbon source, could negatively affect the biomass productivity [23].

3.2. Utilization of SOS as a Carbon Source

Once the optimal growth condition for *A. mangrovei* RCC893 was established, the carbon content provided by the glucose (in the standard media) was substituted at four different degrees to evaluate the utilization of SOS as a main source of organic carbon with respect to the standard conditions with glucose. The results are reported in Figure 3.
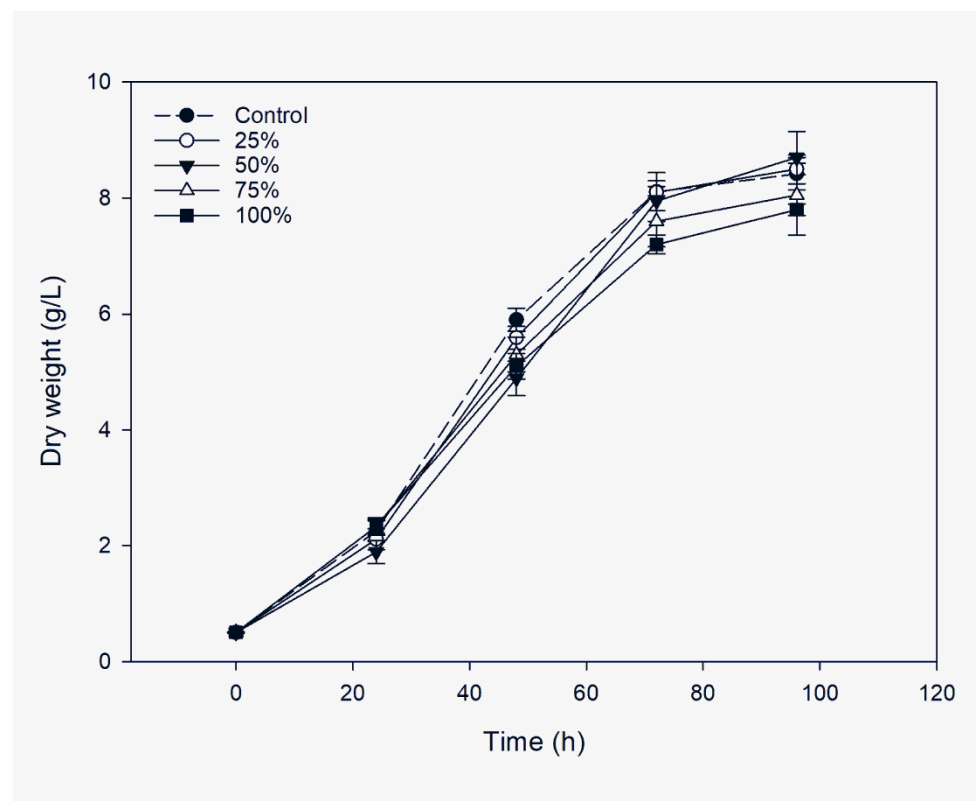

Figure 3. Growth curves of *A. mangrovei* using the new media with progressive substitution (25–100%) of standard glucose with sugars from spent osmotic solution. 100% substitution means that the only organic carbon sources were sugars from SOS.

In terms of biomass, no significant difference respect to the standard media was found from 25 to 100% of substitution of glucose in the media with sugars of SOS. That proves the capability of this strain to use the nutrients present in SOS as a sole carbon source without any pre-treatment (i.e., sucrose hydrolysis).

Aurantiochytrium species have been tested several times with alternative cheap substrates for their cultivation [24,25]. In particular, Hong et al. (2011) obtained a biomass productivity of 16.7 g L^{-1} day^{-1} for Aurantiochytrium sp. KRS101 in fed-batch mode

using sugar cane molasses instead of glucose [24]. Iwasaka et al. (2013) cultivated Aurantiochytrium sp. KH105 using waste syrup from the fruit industry to obtain DHA and astaxhantin [26]. The authors optimized the waste concentration with CCD to obtain a DW of 8.5 g L^{-1}.

Molasses-like substrates resulted in a very interesting alternative to pure glucose as a nutrient source, but inhibitory substances in these by-products must be considered in order to increase the biomass productivity [25]. In our study, SOS did not show any particular inhibitory effects, even at 100% substitution of glucose in the standard media.

3.3. Response Surface Results for Biomass and DHA Productivity

The response surface method was used to optimize the ratio between SOS concentration and yeast extract to assess the best biomass and DHA productivity at different C/N ratios. Experimental design was performed using CCD; fourteen sets of experiments at different concentrations were performed in duplicate to obtain the mean values. In Table S1, the experimental factors, the responses and the predicted values for biomass and DHA productivity are reported. The experimental results for biomass and DHA productivity are comparable to the predicted values.

The significance of the model was tested by ANOVA for biomass (Table S2) and DHA (Table S3) productivity, and a *p*-value lower than 0.05 was considered significant in the analysis.

In the model fit for biomass productivity, the coefficient of determination (R^2) was 0.981, indicating that 98.1% of the variability in the Y (response) could be explained by the model. The *p*-value of the model was $p < 0.001$ and the lack of fit was not significant ($p > 0.05$), proving the validity of the model.

The significance of the model for DHA productivity was 0.001, indicating that the model was highly significant. Moreover, the lack of fit was not significant ($p = 0.921$) and the R^2 of the second-order polynomial prediction equation (3) was 0.943, indicating that the DHA variability can be explained by the model for 94.3% of the total variation.

The experimental results obtained from CCD were regressed using a quadratic polynomial equation, and the regression equations for biomass (2) and DHA (3) productivity are shown below:

$$\text{Biomass productivity } \left(g^{-1}L^{-1}\text{Day}\right)$$
$$= -3.362 + 0.1837\,\text{SOS} + 0.645\,\text{YE} - 0.002360\,\text{SOS} * \text{SOS} \quad (2)$$
$$-0.0332\,\text{YE} * \text{YE} + 0.00111\,\text{SOS} * \text{YE}$$

$$\text{DHA productivity } \left(mg^{-1}L^{-1}\text{Day}\right)$$
$$= -337 + 28.48\,\text{SOS} + 64.2\,\text{YE} - 0.3073\,\text{SOS} * \text{SOS} - 3.65\,\text{YE} \quad (3)$$
$$* \text{YE} + 0.326\,\text{SOS} * \text{YE}$$

Based on an ANOVA analysis, both factors (amount of yeast extract and sugars) showed a significant impact on the biomass productivity of *A. mangrovei*. Figure 4 reports the three-dimensional plot of the response surface results, which allows us to visualize the interactions between factors on the biomass and DHA productivity.

As expected, when the factors were at the minimum level (14 g L^{-1} of sugar from SOS and 4 g L^{-1} of YE), the biomass productivity registered was minimum.

The stationary point for this model was reached at 41.41 g L^{-1} of sugars from SOS and 10.03 g L^{-1} of yeast extract, which are the best conditions to maximize biomass productivity (3.6 g L^{-1} day^{-1}) for *A. mangrovei*. The results show that exceeding a concentration of 60 g L^{-1} of sugars from SOS leads to an inhibition of the growth (Figure 4). This could be explained by the presence of some inhibitory substance (or osmotic stress) that negatively affects the biomass growth. This result is in line with those reported by other authors [9,27,28]. In particular, a study by Nazir et al. (2018) showed an inhibition of biomass growth and DHA production by *Aurantiochytrium* SW1 with a supplementation of fructose higher than 70 g L^{-1} [9]. Nevertheless, *Aurantiochytrium* BL10 was reported to

grow at concentrations of glucose higher than 120 g L^{-1} [29] and 150 g L^{-1} [15], proving that the capacity of substrate consumption differs among the strains of thraustochytrid. The optimal concentration of YE instead is in line with other works that reported the best growth performances at 10 g L^{-1} for *A. mangrovei* [15,30].
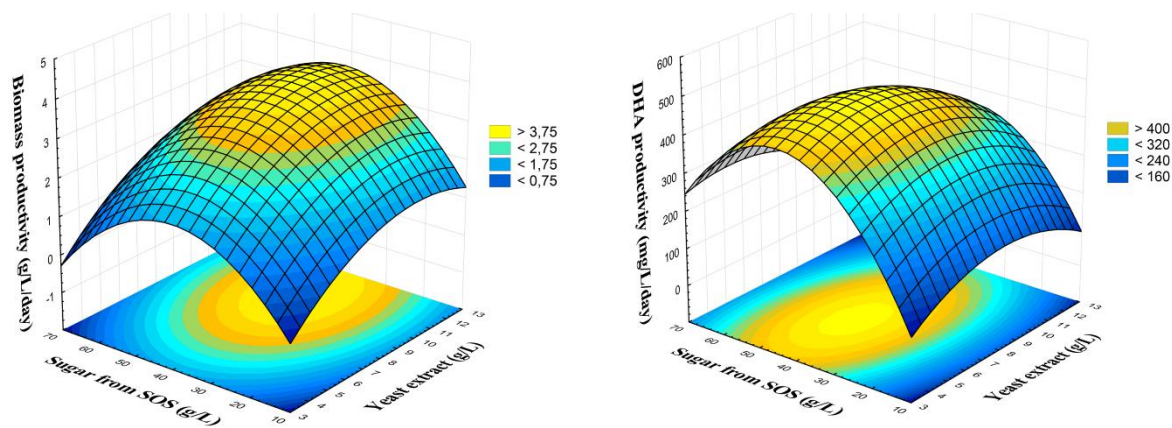

Figure 4. Surface plot for biomass productivity (**left**) and DHA productivity (**right**) using central composite design.

Regarding the interaction between the two factors for DHA productivity, the sugar content was significant ($p = 0.03$) while the YE was not significant ($p > 0.05$). However, from the 3D surface plot (Figure 4) it can be observed that a high concentration of YE leads to lower DHA productivity, while a lower concentration leads to higher productivity. However, N depletion (reduced YE content), as highly reported in literature, leads to an increase in lipid concentration but also results in a higher level of biomass productivity.

Medium concentrations of sugar seem to be optimal for DHA productivity. The DHA productivity obtained in these conditions ranged from 133 to 550 mg L^{-1} day^{-1}. In our experimental results, the best C/N ratio was registered in run 12 (35 g L^{-1} sugar and 8 g L^{-1} YE) with a DHA productivity of 559 mg L^{-1} day^{-1}. The model shows that the maximum predicted DHA productivity (490.9 mg L^{-1} day^{-1}) is reached by means of 42.8 g L^{-1} of sugars and 6.05 g L^{-1} of YE.

Park et al. (2018) obtained a similar DHA productivity ranging from 0.5 to 0.78 g L^{-1} day^{-1} using orange peel extract as a nutrient source. The authors increased the lipid productivity with an addition of glucose in a new formulated media [31]. Liang et al. (2010) also obtained 9.4 g L^{-1} of *Schizochytrium limacinum* using a sweet sorghum juice media, with a DHA productivity of 470 mg L^{-1} day^{-1} [32].

Finally, combining our results for biomass and DHA optimization using a relative regression equation, the best formulation to increase both lipid and biomass productivity resulted in 38.3 g L^{-1} of sugars and 8.7 g L^{-1} of YE. With this formulation, it is possible to obtain a predicted biomass productivity of 3.77 g L^{-1} day^{-1} and a DHA productivity of 475 mg L^{-1} day^{-1}.

3.4. Scale-up Trial

In order to assess the applicability and reproducibility of the optimized media, an upscaled experiment was prepared in 5 L air-lift bioreactor with 3.5 L of working volume using the SOS nutrient recipe with the highest predicted value in terms of both biomass and DHA productivity.

Figure 5 reports the growth curves and sugar consumption during cultivation using an optimized SOS recipe and a control using glucose at the same organic carbon and YE concentrations.
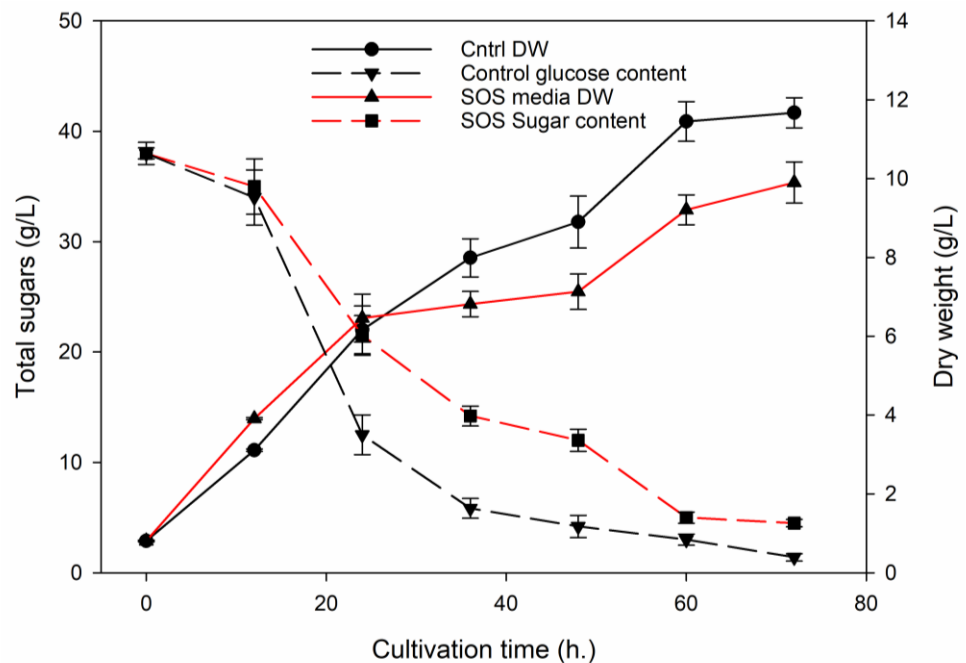
Figure 5. Growth curves and sugar consumption over time for *A. mangrovei* using the SOS optimized media compared to their relative control at the same C/N ratio using glucose. Values are reported as mean ± SD.

The biomass productivity obtained after 72 h was 3.91 ± 0.49 g L^{-1} day^{-1} for the control, as opposed to 3.30 ± 0.24 g L^{-1} day^{-1} for the SOS media recipe. Both the values fit with the predicted growth model developed using the previous experiments.

Sugar consumption followed the biomass increase, with values after 72 h of cultivation lower in pure glucose (2.1 g L^{-1}) than in the SOS recipe (4.6 g L^{-1}).

To better understand the difference between the standard media and the new formulated media, the fatty acid profile was analyzed and is reported in Figure 6.
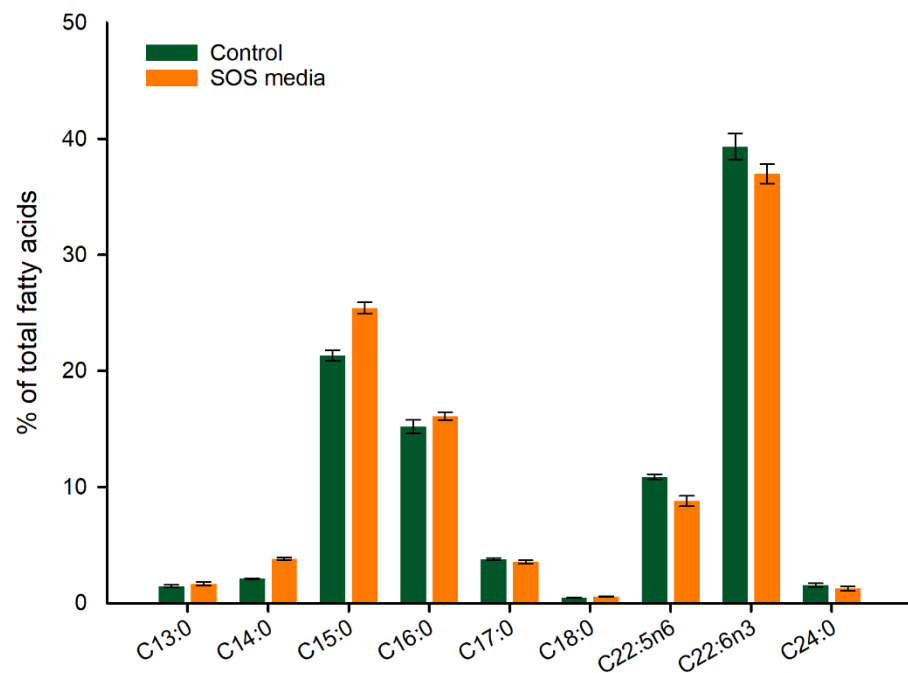

Figure 6. Fatty acid profile (expressed as % of total fatty acids) of the new SOS media compared with the control, using glucose as an organic carbon source. Values are expressed as mean (n = 3) ± SD.

No relevant difference can be observed in terms of lipid profile between the control and the new media. The only significant difference ($p = 0.002$) can be observed for the C15:0, which resulted in a higher value for the SOS media than in the control. Slight differences in DHA and DPA can be observed in the control, respect to the new media. This result is in contrast with another study [15], which obtained a biomass lower than 30% when cultivating *Aurantiochytrium* with only sucrose and 50% lower DHA (with respect to the control with glucose).

However, 36.9% of DHA was obtained in the SOS media, proving the possibility of using the food by-product as a cheap organic carbon source for the production of LC-PUFA.

4. Conclusions

Spent osmotic solution from the candied fruit industry can be employed as an efficient organic carbon source for economical and sustainable DHA production by *A. mangrovei* RCC893. RSM-CCD showed that the best nutrient recipe to maximize both biomass and DHA production was: 41.4 g L^{-1} of sugars from SOS (corresponding to 50 g L^{-1} of SOS) and 8.7 g L^{-1} of YE. The scale-up trial using the optimized condition resulted in a biomass productivity of 3.7 g L^{-1} day^{-1} and a DHA productivity of 475 mg L^{-1} day^{-1}. This alternative media can reduce the production cost of omega-3 oil from algae, with an additional key advantage of recycling a food industry waste, contributing to a sustainable circular economy development.

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