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New Biotechnological Production of EPA by *Pythium irregulare* Using Alternative Sustainable Media Obtained from Food Industry By-Products and Waste

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Abstract: Long-chain polyunsaturated fatty acids (LC-PUFAs) have multiple beneficial effects on human health, in particular docosahexaenoic acid (DHA, 22:6, n-3) and eicosapentaenoic acid (EPA, 20:5, n-3). A variety of microorganisms has been used for the commercial production of non-animal-source LC-PUFAs. The oomycetes of the *Pythium* family are promising EPA producers, and in this work, the optimization of *Pythium irregulare* growth using food industry by-products and wastes as cheap sources of nutrients was carried out. Sugar cane molasses (SCM), spent brewery yeast (SBY), cheese whey (CW), and expired orange juice (EFJ) were tested. A combination of SBY as a source of nitrogen and EFJ as a source of organic carbon resulted in the best outcome among the other sustainable media ingredients. The optimization of the new medium was conducted through a response surface methodology using EFJ and SBY as factors. The results show a significant positive impact of these factors on biomass productivity ($p < 0.005$), with an optimized biomass yield of 14.22 g L^{-1}, a lipid yield of 2.23 g L^{-1}, and an EPA concentration of 155 mg L^{-1}.

Keywords: PUFA; sustainability; food waste; bioconversion; fungi; valorization

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1. Introduction

Omega-3 long-chain fatty acids (n3 LC-PUFAs) represent an important class of compounds from a nutritional point of view. These fatty acids are recognized to be fundamental elements in the human diet, with a series of health effects and for the treatment of cardiovascular diseases, schizophrenia, cancer, and Alzheimer's syndrome [1]. In fact, PUFAs are essential components of cell membranes in neural and muscle tissues and precursors of signaling molecules (bioactive lipid mediators) [2].

The most important LC-PUFAs are: eicosapentaenoic acid (EPA, C20:5), docosapentaenoic acid (DPA, C22:5), and docosahexaenoic acid (DHA, C22:6). The main source of PUFAs for human consumption is fish oil from fatty fishes (i.e., salmon, tuna, herring, anchovies, and mackerel), but this source is not sustainable due to a reduction of global fish stocks, the presence of marine pollutants, and food allergens [3]. Microbial production of PUFAs (in particular from aquatic protists) is a viable alternative to fish oil and represents a sustainable source of DHA and EPA [1]. Therefore, many research groups in recent years have been studying new biotechnological routes to obtain PUFAs. However, the production costs to produce microbial PUFAs remain the main challenge to competing with fish oil [4–6]. In fact, in our previous study, we showed that the medium cost for the production of DHA producer *Aurantiochytrium* sp. accounts for 40% of the total operating costs, and

using agro-food by-products and waste as substitutions for standard stock materials can significantly reduce the production costs of microbial fermentation [5].

Pythium irregulare (phylum Oomycota, kingdom Chromista) is an oleaginous Oomycete with a lipid content between 15 and 20% of dry weight [7,8]. This protist was primarily known as a plant pathogen, causing seedling damping-off and root rot [9,10]. *Pythium* sp. partly shows the lifestyle of fungi, but they are nowadays phylogenetically classified as Stramenopiles, along with brown algae and diatoms [11]. In fact, *P. irregulare* has demonstrated the potential for commercial PUFA production due to its ability to accumulate EPA with a high biomass growth rate [10,12,13].

P. irregulare has shown a high metabolic versatility to use different types of nutrients and grow with food waste-based media. They have been cultivated with corn thin stillage [14], biodiesel-derived crude glycerol [8], sweet whey permeate [15], rendered animal protein [16], soy meal wastewater [12], and soybean processing co-products [13]. Additionally, it was reported to be resistant to a variety of antifungal and antimicrobial compounds [17].

Brewery by-products and waste have shown great potential to replace the nitrogen source in the cultivation of microorganisms, most of all heterotrophic protists [18,19]. Moreover, dairy industry by-products, such as cheese whey, have been extensively studied in the last few years as a new nutrient source for the cultivation of many algae and protists [19,20]. In addition, the utilization of expired shelf date products offers the opportunity to set a circular-oriented bioprocess through their utilization as nutrient sources for new biotechnological processes [21]. An interesting source of nutrients for *P. irregulare* growth could be represented by the recycling of expired fruit juices, which are rich in sugars, vitamins, and organic acids [22]. However, the costs related to the pre-treatments of food waste can affect the economic advantage of using these by-products instead of bulk materials [5]. For this reason, *P. irregulare* could gain more interest due to its capacity to use different sources of organic carbon and nitrogen without any expensive pre-treatments.

To the best of our knowledge, the literature data concerning the growth kinetics of *P. irregulare* are scarce, and, as far as we know, the cultivation of this oomycete using fruit, dairy, and brewery waste as substrate has never been studied.

Therefore, the aim of this work was to research the best growth conditions for *P. irregulare* using a standard medium and alternative food-waste-based media in order to understand the potential to use low-cost nutrient sources for the cultivation of this biomass. Moreover, the EPA production of *P. irregulare* was studied and reported, comparing the standard medium with the new medium from food industry by-products.

2. Materials and Methods

2.1. Organisms and Culture Conditions

The oomycete *Pythium irregulare* (Leibniz Institute, Culture collection DSMZ, strain number DSM62956, transfer date 28 March 2021) was used for the experiments. For the maintenance of the cultures, V-8 JUICE AGAR was used as specified by the strain provider (see https://bacmedia.dsmz.de/medium/310 (accessed on 9 May 2022) for details). Agar plates were incubated at 24 °C and renewed every 15 d. Liquid cultures were performed by placing 4 mm^2 of a petri dish culture in 1.5 mL of a 12-well plates using V-8 JUICE medium and modified YEP medium (20 g/L of glucose, 2 g/L of yeast extract, 2 g/L of peptone, 200 mg/L of K_2HPO_4, 5 mg/L of Fe-EDTA, and 1 mL/L of A5 trace element solution [19]). The pH of the medium was adjusted to 7.2 before autoclaving at 121 °C for 15 min. Modified YEP medium showed better growth results with respect to V-8 JUICE, with cleaner biomass production, so it was used as the standard medium.

For the following experiments of culture optimization, the biomass contents of each microwell, accounting for about 2 ± 0.2 mg of dry weight (DW), were inoculated in 50 mL Erlenmeyer flasks with 20 mL of modified YEP medium and placed in climatic chambers on an orbital shaker at 100 rpm.

2.2. Determination of Growth Kinetics

The biomass dry weight (DW) was used as the main growth parameter. The *Pythium* biomass from each flask was harvested, vacuum-filtered using a Whatman no. 1 filter, and rinsed with deionized water in order to remove residual mineral salts from the culture medium. The biomass was then transferred to a pre-weighted plate and dried in a drying tunnel at 50 °C until constant weight.

2.3. Organic Carbon and Nitrogen Source Screening

For the determination of the carbon source metabolization performances of *P. irregulare*, seven different organic carbons were tested: glucose, lactose, sucrose, galactose, fructose, starch, and glycerol. The amount of C used for each compound was set at 12 g/L for all the trials, corresponding to the standard 30 g/L of glucose used in the control medium. A negative control was also used to evaluate the growth without the addition of any organic carbon.

To study the growth of *P. irregulare* with different sources of nitrogen, we used 6 nitrogen compounds: monosodium glutamate (MSG), ammonium sulfate ($(NH_4)_2SO_4$), potassium nitrate (KNO_3), urea, yeast extract, and peptone. The amount of N was the same for all the samples, 765 mg L^{-1}, corresponding to the amount of nitrogen in the modified YEP medium used as the control medium. The organic carbon source used was glucose at 30 g/L.

2.4. Temperature Test and Saline Concentration Screening

To determine the optimal growth temperature, five different temperatures were tested: 17, 21, 25, 29, and 33 °C. The experiments were conducted in 50 mL Erlenmeyer flasks with modified YEP medium, the temperature was maintained by a climatic chamber, and shaking was provided by an orbital shaker set at 85 RPM. For the evaluation of saline concentrations on *P. irregulare* growth, different amounts of NaCl were added to the modified YEP medium, and experiments were performed at 25 °C. The concentrations of NaCl used were: 0, 4, 8, 16, and 32 g/L, supplementing the standard medium.

2.5. Food Waste Trials

In order to evaluate the utilization of various food by-products and waste (FBW), a screening test was carried out to replace the standard nutrients with a combination of different FBW. The food wastes used were: spent brewery yeast (SBY) from brewery manufacturing, cheese whey (CW) from mozzarella cheese production, sugar cane molasses (SCM), corn steep liquor (CSL), and expired orange juice (EFJ). The CW and SBY pre-treatments and proximal compositions were reported in our previous work [19]. The SBY was hydrolyzed following the standard autolysis in distilled water method reported by Jacob et al., 2019 [23], while the CW was neutralized at pH 7 and heated at 80 °C in order to separate the precipitate from the CW. The sugar cane molasses and CSL were purchased from Merck and used without any pretreatment. The EFJ was generously provided by a local market. The EFJ sugar composition was 141 ± 2 g L^{-1} of total sugars, of which 59% were non-reducing sugars (sucrose) and 41% were reducing sugars (glucose and fructose).

The food waste screening was performed with different combinations of FBW, in order to obtain the optimal combination of an organic carbon source (SCM, CW, EFJ, CSL) and a nitrogen source (SBY, CW, CSL). The selection of the FBW was conducted after our previous experience with these types of substrates on aquatic protist cultivation [19,24]. Specifically, we prepared four formulations: Medium A (TA), with 45 g L^{-1} of sugar cane molasses and 9.5 g L^{-1} of CSL; Medium B (TB), with 600 mL L^{-1} of CW (60% *w*/*v*) and 9.5 g L^{-1} CSL; Medium C (TC), with 230 mL L^{-1} of EFJ (43.3% *w*/*v*) and 7 g L^{-1} of SBY; Medium D (TD), with 45 g L^{-1} of SCM and 7 g L^{-1} of SBY. The formulations were made in order to obtain the same amount of organic carbon and nitrogen as the standard YEP medium.

In the preparation of the alternative media, deionized water was used; the solutions were adjusted to pH 7 and sterilized in an autoclave prior to their utilization.

2.6. Response Surface Methodology

The food waste medium was optimized using response surface methodology (RSM) as previously performed in other fermentation works [19,25–28]. This method was applied to formulate the optimal combination of EFJ and SBY as organic carbon and nitrogen source, respectively. SBY supplementation was expressed as g L^{-1} DW of dried extract.

The RSM was carried out by constructing a five-level full factorial central composite design (CCD). The design included star points, which represent extreme values ($-\alpha$, α) for each input factor. The coded value of alpha depends on the number of factors and as such, $\boldsymbol{\alpha} = \sqrt{\mathbf{k}} = 1.414$, considering that k corresponds to the number of factors used in this design. The mathematical relationship of the response (Y) to the significant independent variables X_1 and X_2 is given by the following quadratic polynomial Equation (1):

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

where Y is the predicted response; X_i and X_j are the coded values; β_0 is 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 of the interaction and quadratic effects, respectively [19].

The experimental setup is reported in Table 1.

Table 1. Experimental design and results of biomass growth optimization with supplementation of sugars from EFJ and SBY by central composite design.

Run	Factor Assignment		Biomass Dry Weight (Y)	
	X_1	X_2	Experimental Value (g L^{-1})	Predicted Value (g L^{-1})
1	0	0	13.94	13.58
2	0	0	13.1	13.58
3	$-\alpha$	0	11.69	12.17
4	−1	+1	12.6	12.42
5	+1	−1	8.01	6.9
6	0	0	13.55	13.58
7	0	0	14.75	13.58
8	0	$-\alpha$	6.88	7.96
9	0	$+\alpha$	10.25	10.49
10	+1	+1	8.57	7.93
11	−1	−1	11.04	10.16
12	0	0	12.98	13.58
13	$+\alpha$	0	5.85	6.48

Coded values: X_1 = sugars from EFJ (g/L); X_2 = SBY (g/L); the five levels ($-\alpha$, −1, 0, +1, $+\alpha$) set for sucrose were 26.87, 69.3, 99.3, 111.73 g L^{-1}, while for SBY, they were 0.715, 3.2, 9.2, 15.2, and 17,68 g L^{-1}.

2.7. Lipid Extraction and Fatty Acid Analysis

Lipid extraction and the transmethylation of fatty acids to obtain the methyl-esters of fatty acids were performed according to the method used by Russo et al. 2021 [19]. FAMEs were separated in a gas chromatography–mass spectrometer (GC-MS) using the method proposed by Conde et al. (2021) [29]. The equipment consisted of an Agilent 7890A gas chromatographer coupled to a Waters QUATTRO microTM mass spectrometer detector. The separation was performed on a DB-5MS (30 m × 0.25 mm; f.t. 0.25 µm) from Agilent Technologies (J&W Scientific, Folsom, CA, USA). The oven temperature was 58 °C for 2 min, 25 °C min^{-1} to 160 °C, 2 °C min^{-1} to 210 °C, 30 °C min^{-1} to 225 °C (held for 20 min). The MS detector was operated with an ionization energy of 70 eV and a scanning range of m/z 50–550 m/z. The conditions were helium as the carrier gas at 1.4 mL min^{-1}, an inlet temperature of 220 °C, detector temperature of 230 °C, and 2 µL of injection volume (splitless). The data were analyzed using MassLynx version 4.1 (Waters, San Jose, CA, USA).

2.8. Statistical Analysis

All the analyses were carried out in triplicate, and the average values with standard deviation were reported. One-way ANOVA was applied using the raw data to test for

significant differences among the samples (the significance level was always set at $p < 0.05$). Tukey's test was used for post-hoc analysis when there were significant differences among the samples. The data were analyzed using IBM© SPSS© Statistics software Ver. 23 (SPSS, Inc., Chicago, IL, USA). RSM analysis was carried out using the Statistica 7.0 package (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Effect of Different Sources of Organic Carbon

The growth performances of *P. irregulare* cultivated with six different sources of organic carbon were determined, and the results are presented in Figure 1.

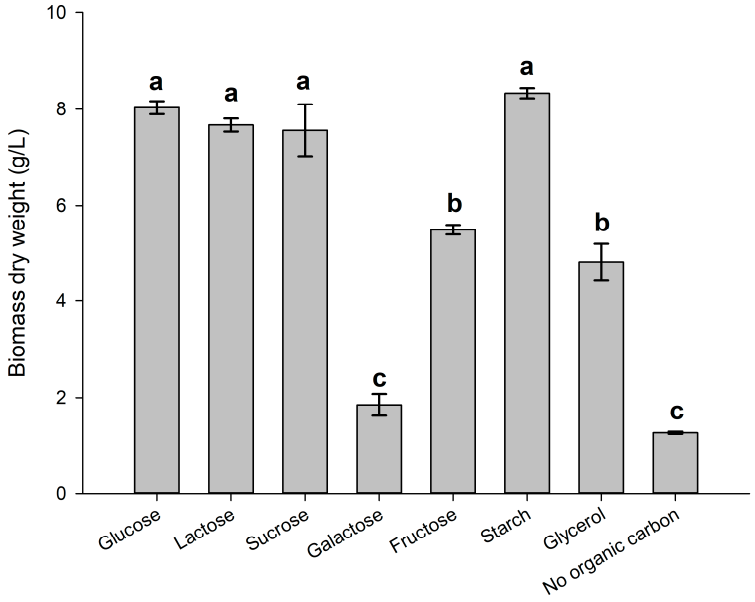

Figure 1. Screening test for different types of organic carbon sources for *P. irregulare* cultivation. Different letters mean a significant difference ($p < 0.05$).

The highest biomass after 7 d of cultivation was obtained with the medium supplemented with glucose, lactose, sucrose, and starch, reaching a biomass DW of 8–8.2 g/L. Glucose is currently the monosaccharide of choice for *P. irregulare* cultivation. Nevertheless, our results suggest that lactose, sucrose, and starch can also be effectively used as substitutions for glucose without a significant difference in terms of biomass growth. In fact, lactose was used in another study with *P. irregulare* [15], which showed good growth performance with a lactose-based medium. Moreover, sucrose was successfully used for the cultivation of another *Pythium specie* (*P. ultimum*), which is in line with our experiment [30].

Pythium species (like all *oomycetes* species) are capable of degrading carbohydrate polymers, in particular, cellulose and starch from plant roots [31,32]. In fact, *P. irregulare* grew well with the corn starch used in our experiment, reaching a biomass DW of 8.22 g/L at the end of the cultivation time. This is in line with another study that evaluated the sucrose and starch catabolism by different species of *Pythium* and also reported a high number of carbohydrate-active enzymes, including polysaccharide lyases and glycoside hydrolases [31].

Instead, the medium with fructose and glycerol supplementation showed a significantly lower biomass (4.5–5.5 g/L of DW) with respect to the organic carbon mentioned above. Glycerol was also used in the work of Liang et al. (2012) [16] for the growth of *P. irregulare*, obtaining a dried biomass of 4.8 g/L and reporting, as in our study, significantly reduced biomass growth compared to the other media. Other authors also reported a lower growth of *P. irregulare* when cultivated with crude glycerol compared to glucose [8].

In other words, galactose did not show any significant growth with respect to the negative control (a medium without supplementation of organic carbon), which suggests

an inability to metabolize this monosaccharide. In fact, O' Brien et al. also reported that *P. irregulare* was unable to metabolize pure galactose [15].

Once the organic carbon screening was performed, the biomass conversion yield of glucose was assessed using increasing concentrations of glucose in the standard medium. Figure 2 shows the results of the growth performances at different concentrations of glucose with the relative efficiency of use.

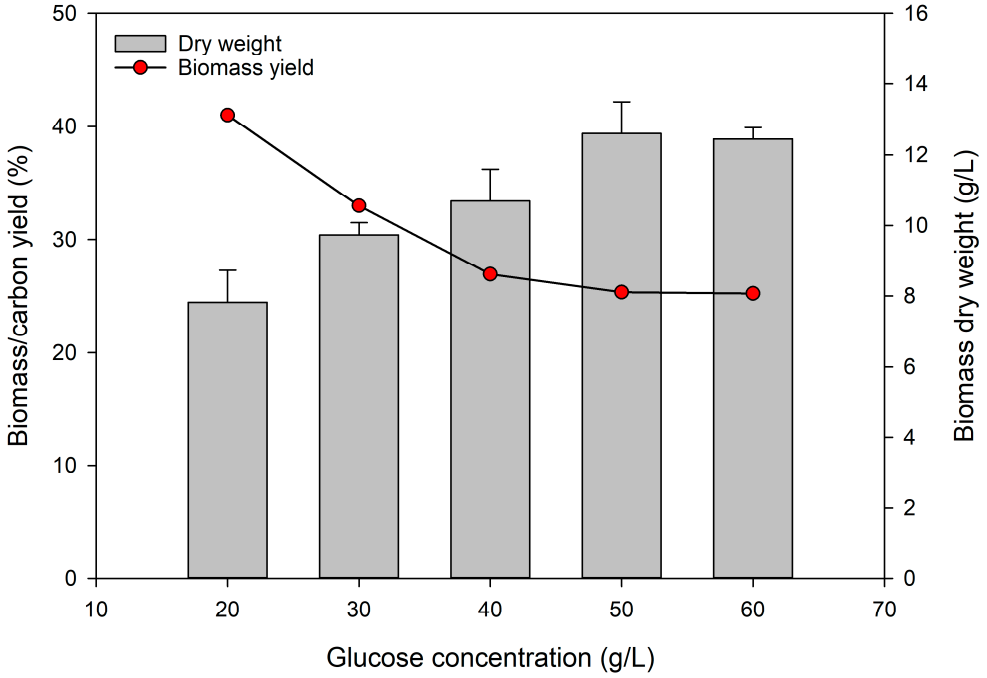

Figure 2. Biomass/carbon conversion rate and biomass dry weight of *P. irregulare* after 7 d of cultivation using different concentrations of glucose in standard medium.

The highest carbon/biomass conversion efficiency (41%) was achieved with 20 g L^{-1} of glucose. However, the dried biomass obtained was the lowest with respect to the other formulations. The highest biomass DW was obtained with 50 g L^{-1} of glucose, and further carbon additions did not show any significant improvement in growth performance. The conversion efficiency is an important factor to study in order to achieve the highest biomass obtainable at the lowest supplementation of organic carbon. This yield is mostly important for further scaling up in order to reduce production costs and thus avoid wasting resources [24]. In our case, the best compromise would be to use 30 g L^{-1} of glucose, thus obtaining a DW of about 10 g L^{-1}, rather than using 50 g L^{-1} of glucose to obtain just an increased DW of 2 g L^{-1}.

Thus, subsequent experiments were conducted using 30 g L^{-1} of glucose.

3.2. Evaluation of Different Sources of Nitrogen on Growth Performances

The results of the nitrogen source screening are reported in Figure 3.

The optimal N sources for *P. irregulare* were MSG, yeast extract, and peptone, with a DW higher than 10 g L^{-1}. Potassium nitrate, urea, and ammonium sulfate instead showed significantly lower biomass production with respect to the above-mentioned nitrogen sources. As for the organic carbon, no previous studies on *Pythium irregulare* have been found in the literature for the evaluation of the optimal nitrogen source. However, it was reported that nitrate, ammonium, and nitrite can be used as nitrogen sources by *P. irregulare* in the glutamate, glutamine, proline, and arginine pathways, which are derived from 2-oxoglutarate after the TCA cycle [32]. Nevertheless, minor expressions of nitrate reductase activity and ammonium transporters have been reported for similar oomycetes

species [33,34], which could explain why nitrate showed significantly lower biomass production with respect to protein and amino acid nitrogen.

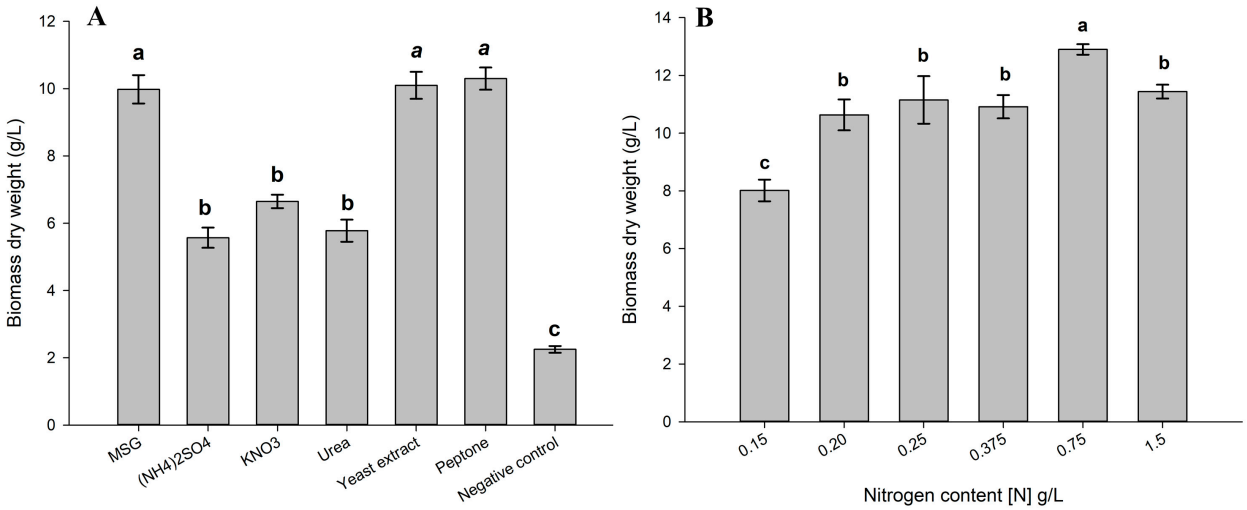

Figure 3. Screening test of different sources (**A**) and concentrations (**B**) of nitrogen on *P. irregulare* growth. The organic carbon source used in the experiment was glucose (30 g L^{-1}). Different nitrogen concentrations in (**B**) were achieved using yeast extract. Data are expressed as mean ± SD. Different letters mean a significant difference ($p < 0.05$).

In the case of yeast extract, peptone, and MSG, the supplementation of hydrolyzed peptides and amino acids in the medium seemed to maximize biomass production. This suggests a faster uptake of organic nitrogen (in particular amino acids and small peptides) with respect to nitrate and ammonium. It was reported that during the early stages of growth, *Pythium* sp. showed an overexpression of genes associated with the ATP-binding cassette (ABC) and transmembrane transporters, including amino acid/auxin permeases (AAAP) [34].

With these assumptions, it is possible to explain the biomass growth differences between inorganic and organic nitrogen supplementations. In the case of urea, on the other hand, its utilization by *P. irregulare* resulted in less efficiency than other sources of organic nitrogen. In fact, in the literature, it was reported that most *Pythium* species have the necessary urease, but accessory proteins are poorly expressed, especially for *P. irregulare* and *P. ultimum* [35].

After this screening, we decided to use yeast extract as the optimal nitrogen source for *P. irregulare*.

The optimal nitrogen concentration to be supplied to the medium was also evaluated, and the results are reported in Figure 3. The highest biomass DW was obtained with a supplementation of 0.75 g/L of N, corresponding to 7 g L^{-1} of yeast extract. In general, we observed that the nitrogen requirement of *P. irregulare* was not high as the organic carbon source. In fact, even with the supplementation of 0.15 g L^{-1} of N, the biomass showed significant growth (8.1 g L^{-1} of DW). Moreover, significant growth of *P. irregulare* even with only 2 g L^{-1} of yeast extract was previously reported in another study [7]. This is a positive finding, as less nitrogen supplementation would reduce the production costs for biomass cultivation.

3.3. Effects of Temperature and Salinity on Biomass Growth

The effects of different temperatures were also investigated, and the results are reported in Figure 4.

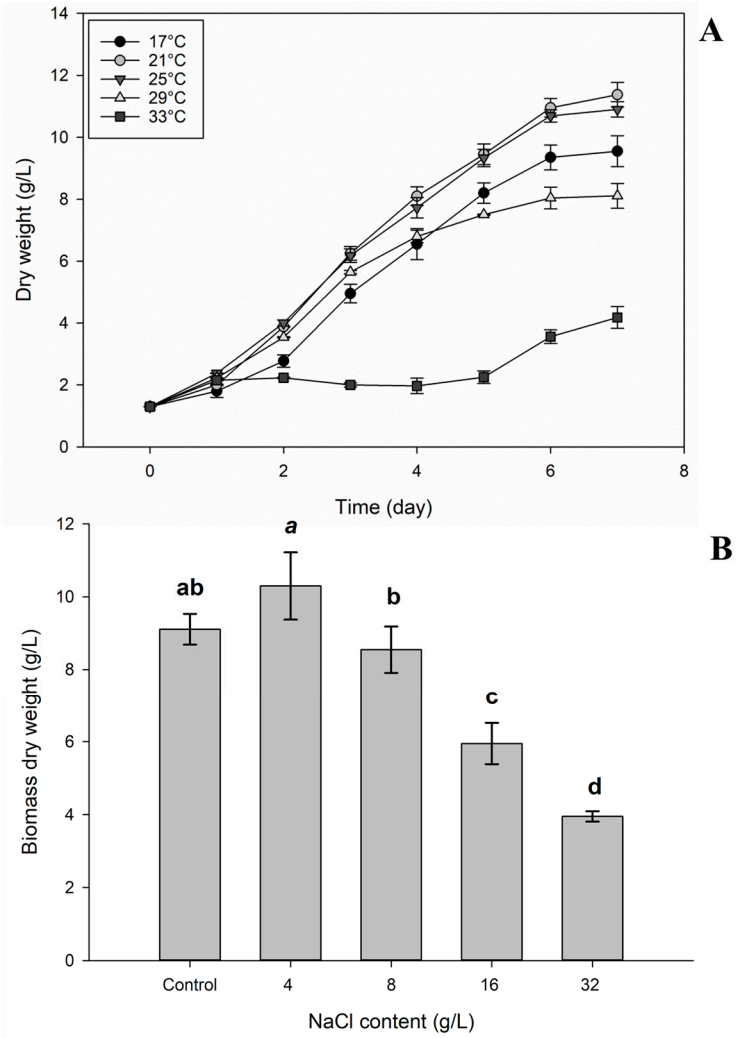

Figure 4. Effects of temperature (**A**) and NaCl supplementation (**B**) on growth performance of *Pythium irregulare* (control without NaCl supplementation). Data are expressed as mean (n = 3) ± SD. Different letters mean a significant difference ($p < 0.05$).

The best growth performances were obtained at 21 and 25 °C, with final biomass dry weights that were not statistically different, at 10.9 and 10.8 g L^{-1}, respectively. According to the literature, *Pythium* sp. is able to grow at a high-temperature range (15–30 °C). Cold temperatures have been reported to be the best condition to maximize the PUFA contents in *P. irregulare* [14]. However, in our experiments, we obtained the highest biomass at 21 °C, while the culture growth at 17 °C was significantly lower than the cultures at 21 and 25 °C. Athalye et al. (2009) [8] reported *P. irregulare* grown at 25 °C, while in the work of O' Brien et al. (1993) [15] the protist grew at 22 °C and 14 °C, reporting a significantly higher content of EPA at the low temperature. However, the authors reported that the carbon consumption and biomass production were 50% lower at cold temperatures, affecting the EPA productivity.

In a recent study on P. splendens RBB-5, the optimal growth temperature was 25 °C (in line with our study), but the authors reported lower lipid productivity in this condition [36]. In fact, it is well known that in aquatic protists and fungi, a cold temperature stimulates the expressions of elongase and desaturase enzymes [1,15,36]. However, in the case of *P. irregulare*, this is difficult to establish, as the growth conditions (growth medium composition, fermenters, shake flask, etc.) are often very different among the various published works.

To investigate the best growth conditions and with a view to using FBW-based media, we also evaluated the growth of *P. irregulare* at various saline concentrations. Figure 4 shows the growth performance of *P. irregulare* at different concentrations of NaCl. The control used was the standard medium without NaCl. We did not observe any significant differences between 0 g L^{-1} and 8 g L^{-1} of NaCl supplemented to the growth medium. However, a saline concentration higher than 8 g L^{-1} showed an inhibitory effect on the biomass growth. Nevertheless, *P. irregulare* showed a good level of robustness in terms of saline tolerance, as we observed growth of this oomycete even at 32 g L^{-1} of NaCl, which is almost the saline concentration of seawater. Tolerance to osmotic stresses is an important characteristic for an organism that needs to be grown on FBW. In fact, many FBWs have in common a high saline content, which is one of the main causes that make food processing wastewater hard to use for biotechnological purposes [1].

Knowing all these factors, *P. irregulare* seems to be a great candidate for using FBW as a new sustainable growth medium.

3.4. Screening of Food Waste Medium

Figure 5 shows the results of the new sustainable media from FBW.

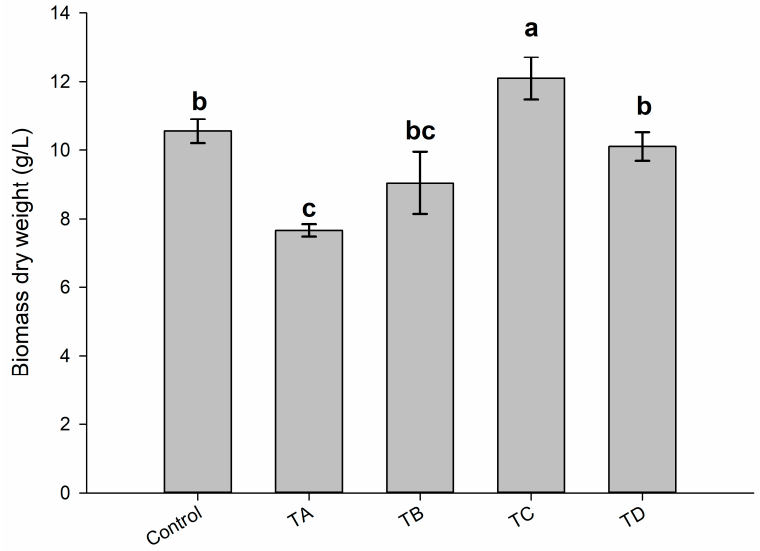

Figure 5. Growth performances of the new food-waste based media on *P. irregulare*. TA = medium with sugar cane molasses and corn steep liquor; TB = medium with cheese whey and corn steep liquor; TC = medium with expired fruit juice and spent brewery yeast; TD = medium with sugar cane molasses and spent brewery yeast. Data are expressed as mean ± SD. Different letters mean a significant difference ($p < 0.05$).

The TC medium, which was based on EFJ and SBY, resulted in the best formulation, achieving a biomass of 12.11 g/L after 7 d of cultivation. The TB and TD media obtained results comparable to the standard control, while the worst growth performance was observed with the medium based on SCM and CSL (TA), which showed a significantly lower biomass with respect to the control and the other formulations. This difference in growth performance was probably due to the different sources of organic carbon and nitrogen. In fact, in the CW-based medium, lactose was the predominant organic carbon source, while in the case of EFJ and SCM, sucrose was the most present carbon. Nevertheless, significant biomass growth was observed in all tested media, further highlighting the ability of *P. irregulare* to grow with different types of nutrients. The SCM-based formulations, despite the presence of a large amount of sucrose in the molasses, reported lower growth performances with respect to the control. This was probably due to the high presence of calcium and magnesium ions within the molasses, which are known to suppress *P. irregulare*

growth and sporulation [37]. Moreover, as we reported previously, a high saline content negatively affect the growth of this protist (Figure 4).

From this screening, it is evident that EFJ is a promising nutrient source to significantly increase the biomass productivity of *P. irregulare*. In Europe, it has been estimated that up to 8.8 million tons of food are wasted annually in the European Union due to date labeling [38]. Nevertheless, expired foods can be a valuable source of nutrients for biotechnological use, most of all for the growth of fungi and oomycetes [21]. The EFJ used in this study was very rich in sugars (mainly sucrose and fractions of glucose and fructose) that can be easily uptaken by *P. irregulare*, as we stated before. Moreover, the presence of ascorbic acid, folate, and organic acids present within the fruit juice [22] may be growth promoters for *P. irregulare*, which would explain the higher growth with respect to the control.

Costs related to feedstock and raw materials have a great impact on the overall economics of heterotrophic biomass cultivation, and finding cheaper sources of carbon and an optimal amount to supply is a necessary step to reducing production costs [5]. For this, the use of alternative sources of low-cost nutrients, such as waste and by-products from the food industry, would make the cultivation of protists and omega-3 production more economically sustainable [39].

3.5. Food-Waste Medium Optimization with RSM

The ANOVA results of RSM-CCD using EJF and SBY as factors are reported in Table 2.

Table 2. Analysis of variance for biomass production using coded values and regression equation.

Source	DF [a]	Adj SS [b]	Adj MS [c]	F-Value	*p*-Value
Model	5	96.076	19.2153	16.32	0.001
Linear	2	38.801	19.4005	16.48	0.002
EFJ (X1)	1	32.361	32.3608	27.49	0.001
SBY (X2)	1	6.440	6.4402	5.47	0.041
Square	2	57.060	28.5298	24.23	0.001
EFJxEFJ	1	31.486	31.4859	26.74	0.001
SBYxSBY	1	33.014	33.0136	28.04	0.001
2-Way Interaction	1	0.216	0.2158	0.18	0.681
EFJxSBY	1	0.216	0.2158	0.18	0.681
Error	7	8.242	1.1774		
Lack-of-Fit	3	6.925	2.3083	7.01	0.095
Pure Error	4	1.317	0.3291		
Total	12	104.318			

R^2 = 95.22 ([a] DF, degree of freedom; [b] SS, sum of squares; [c] MS, mean squares; F, probability of distribution; *p*, probability).

The RSM model previously described resulted significant to the analysis with $p = 0.001$. The variables analyzed were: the concentration of sugars from EFJ (expressed in g/L) and the concentration of SBY lysate supplemented to the medium. The lack of fit was not significant ($p = 0.095$) and the R^2 was higher than 95%, proving the significance of the whole model and its robustness.

The experimental values obtained from the central composite design (CCD) were regressed using a quadratic polynomial equation, and the regression Equation (2), expressed in terms of the actual factors, is shown below.

$$\begin{gathered} Biomass\ dry\ weight\ \left(gL^{-1}\right) = -0.44 + 0.2725\ EFJ + 1.352\ SBY \\ -0.002364\ EFJXEFJ - 0.0605\ SBYXSBY - 0.00129\ EFJXSBY \end{gathered} \quad (2)$$

The interaction between the factors did not show any significant impacts on biomass production (Table 2). However, since the individual effects of SBY and EFJ supplementation showed significant impacts on biomass productivity, one factor plot for these two factors was generated to better understand the results from the RSM analysis (Figure 6).

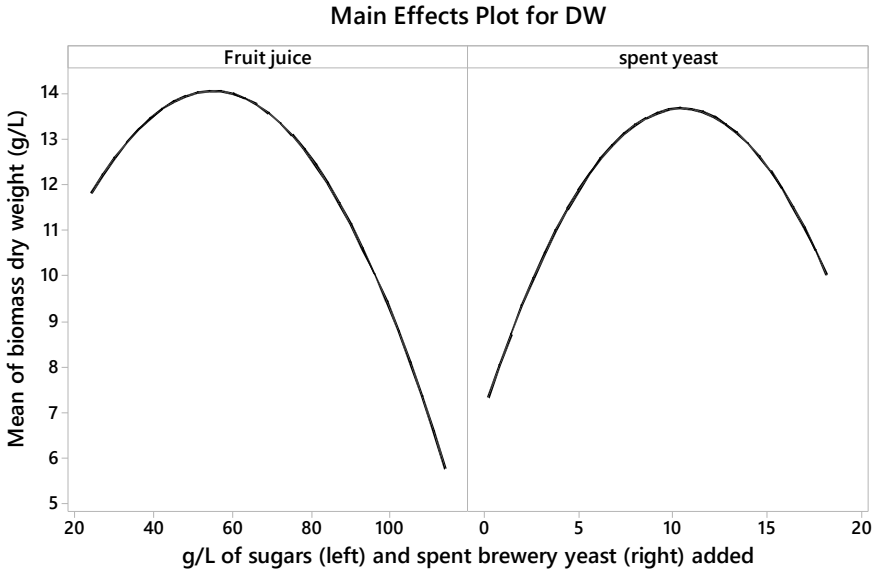

Figure 6. Main effect plot of sugars from EFJ (**left**) and SBY supplementation (**right**) on biomass production of *P. irregulare*.

Based on Figure 6, the EFJ sugar increase from 20 g/L to 55 g/L had a positive influence on *P. irregulare* production. This is in line with our previous screening (Figure 2), confirming the optimal utilization of the sucrose present in EFJ by *P. irregulare*. However, the results show that an increase in the sugar concentration above 60 g/L was deleterious for biomass productivity, with a significant reduction in growth performance. This was most probably due to the inhibition of sporulation by a high concentration of mineral salts and sucrose, as already reported by our previous experiment and from the literature data [37,40]. In fact, a reduced sporulation rate directly affected the mycelium growth in some species of *Pythium* [40] This result suggests that SBY is a favorable nitrogen source in enhancing the biomass production of *P. irregulare.* Nevertheless, regarding the EFJ concentration, a high amount of SBY supplemented to the medium led to lower biomass productivity.

The effects of different concentrations of SBY have never been tested before on the growth of this protist. However, in our previous study, on the thraustochytrid *Aurantiochytrium mangrovei,* we also reported a positive effect of SBY supplementation on the culture medium [19]. In fact, in our previous study, we observed that SBY extracts can integrally substitute the standard nitrogen source for the cultivation of heterotrophic protists. This is also in agreement with *P. irregulare*.

Based on the regression analysis of the model equation for biomass production, the optimum levels of the variables were estimated. The optimum conditions were 52 g L^{-1} of the sugars from EFJ and 9.2 g L^{-1} of SBY (corresponding to 0.87 g L^{-1} of nitrogen) supplemented to the medium in order to obtain a dried biomass of 14.22 g L^{-1}.

3.6. Effect of New Sustainable Medium on Fatty Acid Production

After the screening and optimization with alternative food waste media, we performed an analysis of the fatty acids of *P. irregulare* at the end of the cultivation time. The results are reported in Table 3.

The main components of the oil fraction analyzed were oleic acid (C18:1) and palmitic acid (C16:0). Some significant differences were detected between the samples, especially for the palmitic acid, myristic acid (C14:0), linoleic acid (C18:2), and EPA. The EPA obtained among all the samples ranged between 6.9% and 8.4% on the total fatty acids (TFAs).

Table 3. Fatty acid profiles (g/100 g) on the lipids extracted from *P. irregulare* grown on EFJ medium and EFJ medium optimized after response surface.

Fatty Acids	Control	EFJ Medium	EFJ Optimized Medium
C14:0	6.86 ± 0.05^{b}	5.38 ± 0.1^{b}	8.64 ± 0.12^{a}
C16:0	19.05 ± 0.45^{ab}	21.66 ± 1.02^{b}	24.12 ± 0.22^{c}
C16:1	5.82 ± 0.37^{b}	8.92 ± 0.78^{a}	8.94 ± 0.31^{a}
C18:0	9.71 ± 0.11^{a}	6.74 ± 0.15^{c}	8.30 ± 0.28^{b}
C18:1	22.91 ± 0.27^{a}	23.01 ± 0.74^{a}	24.02 ± 1.08^{a}
C18:2	9.85 ± 0.31^{ab}	10.81 ± 0.38^{b}	7.64 ± 0.26^{a}
C20:3	3.81 ± 0.33^{a}	3.17 ± 0.13^{a}	3.14 ± 0.09^{a}
C20:4	3.80 ± 0.14^{a}	3.18 ± 0.23^{a}	2.45 ± 0.12^{a}
C22:1	2.06 ± 0.23^{a}	1.53 ± 0.07^{a}	2.14 ± 0.08^{a}
C20:5 EPA	6.92 ± 0.37^{ab}	8.24 ± 0.44^{c}	7.86 ± 0.21^{b}
Σ PUFAs	24.08	25.4	21.09
Total lipids (g L^{-1})	1.43 ± 0.14^{a}	1.66 ± 0.05^{a}	2.25 ± 0.16^{b}
EPA yield (mg L^{-1})	96 ± 1.25^{b}	136 ± 3.45^{a}	176 ± 4.12^{c}

Values are expressed as means ± SD (n = 3). PUFAs = polyunsaturated fatty acids; EFJ = expired fruit juice. Values followed by different letters on the same line are significantly different ($p < 0.05$).

P. irregulare EPA production was maximized when cultivated in the EFJ medium, obtaining a significantly higher yield with respect to the standard medium (176 mg L^{-1} vs. 96 mg L^{-1}). This was probably due to the different sources of organic carbon and nitrogen with respect to the control. Additionally, between the two media, there was a different C/N ratio, which could have affected the biomass and lipid productivity. In fact, the standard YEP medium had a C/N of approximately 15, while the new optimized medium had a C/N ratio of 23. The effects of different C/N ratios on *P. irregulare* were studied in the work of [16]. The authors reported that despite a nitrogen starvation with a high C/N ratio, the oomycete did not demonstrate an increase in lipid productivity, as the lipid synthesis is species-specific. Moreover, they stated that a different amino acid quality of the growth medium significantly affected the lipid productivity of *P. irregulare*. In our case, the SBY used as the sole nitrogen source increased the biomass and EPA yield with respect to the standard medium.

The EPA yield found in our study was higher than in the study of Athalye et al. (2009), in which *P. irregulare* was cultivated using crude glycerol from biodiesel production [8]. In that case, the utilization of glycerol negatively affected biomass production, leading to a lower EPA production with respect to our work. In the study of Liang et al. (2011) [16], instead, the EPA production was comparable to ours despite the utilization of a different nitrogen source (rendered animal proteins).

P. irregulare has been shown to be an excellent producer of EPA compared to other microbial sources, such as marine diatoms. For example, in the study of Wang et al. (2018) [41], the authors reported EPA yields of 62.55 and 27.32 mg L^{-1} for the diatoms *Phaeodactylum tricornutum* and *Cylindrotheca fusiformis*, respectively.

Nevertheless, the EPA yield obtained with *P. irregulare* in our work was significantly lower compared to another study on the oleaginous fungus *Mortierella alpina* (1.01 mg L^{-1}) [42]. However, optimization of the lipid yield was not examined in our study; only that of biomass productivity on food waste-based medium was determined.

The composition of *P. irregulare* biomass highly depends on the nutrients used in the medium, as many authors have suggested [16,32]. Moreover, biosynthetic pathways leading to the formation of EPA and its metabolites in *Pythium* species still remain unclear [16]. However, in 2019, Fernandes et al. [32] reported the metabolic annotation of *Pythium irregulare* CBS 494.86, proving that this oomycete had biosynthetic fatty acid pathways similar to *S. cerevisiae*, *Y. lipolytica*, and *M. alpine*. In particular, the authors found a gene that encodes the Δ17 desaturase, an enzyme that can use fatty acids both from the acyl-CoA

fraction and the phospholipids fraction as substrates for the synthesis of EPA [43]. This makes the protist an excellent candidate for an efficient producer of omega-3 fatty acids.

4. Conclusions

P. irregulare showed a high ability to grow using different sources of organic carbon and nitrogen. Most of all, FBWs were effectively used as alternative sources of nutrients, and the best formulations were those based on EFJ and SBY. To our knowledge, this is the first time that *P. irregulare* growth with FBW was optimized by RSM-CCD in order to maximize biomass production. The optimization showed that a medium based on 52 g L^{-1} of sugars from EFJ and 9.2 g L^{-1} of SBY led to a dried biomass of 14.22 g L^{-1} and an EPA yield of 176 mg L^{-1}. In this way, it is possible to create new biotechnological processes aimed at using FBW as a low-cost nutrient source for the production of EPA and innovative biomass. Further studies will be needed to evaluate the scale-up of this new biotechnological process. Furthermore, the techno-economic advantages will have to be studied to make the production of microbial EPA by *P. irregulare* more economically sustainable.

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