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Crabtree Effect on *Rhodosporidium toruloides* Using Wood Hydrolysate as a Culture Media

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Abstract: The interest in microorganisms to produce microbial lipids at large-scale processes has increased during the last decades. *Rhodosporidium toruloides*-1588 could be an efficient option for its ability to simultaneously utilize five- and six-carbon sugars. Nevertheless, one of the most important characteristics that any strain needs to be considered or used at an industrial scale is its capacity to grow in substrates with high sugar concentrations. In this study, the effect of high sugar concentrations and the effect of ammonium sulfate were tested on *R. toruloides*-1588 and its capacity to grow and accumulate lipids using undetoxified wood hydrolysates. Batch fermentations showed a catabolic repression effect on *R. toruloides*-1588 growth at sugar concentrations of 120 g/L. The maximum lipid accumulation was 8.2 g/L with palmitic, stearic, oleic, linoleic, and lignoceric acids as predominant fatty acids in the produced lipids. Furthermore, *R. toruloides*-1588 was able to utilize up to 80% of the total xylose content. Additionally, this study is the first to report the effect of using high xylose concentrations on the growth, sugar utilization, and lipid accumulation by *R. toruloides*-1588.

Keywords: substrate inhibition; growth inhibition; wood hydrolysate; lipid production; *Rhodosporidium toruloides*

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1. Introduction

The production of biofuel to replace fossil fuels has increased during the past decades. Among the several routes to produce biofuels, the use of biochemical processes is one of the most attractive due to being more environmentally friendly in comparison with the conventional routes used for fossil fuels [1,2]. Currently, the production of liquid biofuels such as biobutanol, bioethanol, or biodiesel using renewable feedstocks are a potential alternative to conventional fossil fuels that contributes to the development of a sustainable culture and green energy independence [3,4].

Recent research has been focused on producing biodiesel using lipids from several sources such as raw and refined vegetable oil, cooking oils, and animal grease and tallow. Another alternative that has been explored in the last three decades is the single-cell oil from oleaginous yeast [5,6]. Oleaginous yeasts can accumulate 20% to 70% of lipids within their cell. Among these microorganisms, *Rhodosporidium toruloides* stands out as a promising yeast capable of accumulating a high amount of lipids (70%) and can utilize a wide variety of substrates, such as glycerol, food waste-hydrolysates, sodium chloride, and lignocellulosic hydrolysates. They can also degrade several inhibitory compounds that can inhibit microbial growth [7,8]. Numerous studies have reported the performance of *R. toruloides* in terms of the growth, sugar utilization, and lipid production [9–12]. Nevertheless, the differences in the substrates and culture conditions make it difficult to compare those results. For instance, the presence of high sugar concentrations in the culture media

can cause a catabolic repression effect during aerobic fermentation, ultimately hindering the microbial growth, and leading to a decrease in the total lipid accumulation. It has been reported that the excess sugar in the culture media can cause problems during the production of adenosine triphosphate (ATP), a vital compound in the energy metabolism [13,14]. However, some studies have reported that the increase in glucose in oleaginous yeast increased the microbial biomass production and lipid accumulation using a high-sugar culture media such as biomass-based hydrolysates [15,16].

In our previous work, *R. toruloides*-1588 demonstrated an outstanding capacity to use liquid hydrolysates obtained from forestry residues as a suitable substrate for microbial growth and lipid production in the presence of high concentrations of several inhibitory compounds [17–19]. From a viewpoint of the scale-up process, the selection of an optimal sugar concentration is crucial to reach the highest lipid accumulation and prevent the loss of the carbon source during the fermentation. This study aims to analyze the capacity of *R. toruloides*-1588 to grow and accumulate lipids in a high glucose and xylose concentrations using wood hydrolysate as a culture media. Additionally, the study reports the effect of ammonium sulfate as the only nitrogen source on the growth and lipid accumulation.

2. Materials and Methods

2.1. Substrate, Microorganism, and Inoculum Preparation

Hydrolysates were kindly provided by Greenfield Global (Varennes, QC, Canada) which were produced from poplar (*Populus alba*) residues with a glucose-xylose content of 10 g/L and 122 g/L (C5 hydrolysate) and 123 g/L and 11 g/L (C6 hydrolysate). *R. toruloides*-1588 was procured from the Agricultural Research Service (USA) and preserved on YM plates and the inoculum seed was prepared using YM broth [20].

2.2. Sugar Concentration and Ammonium Sulfate Addition Effects

To evaluate the effect of sugar concentrations and ammonium sulfate on the microbial growth, sugar utilization, and lipid production by *R. toruloides*-1588, four initial sugar (glucose for C6 wood hydrolysate and xylose for C5 wood hydrolysate) concentrations (50, 75, 100, and 120 g/L, respectively) and 1 g/L of ammonium sulfate $(NH_4)_2SO_4$ were used. Batch flask fermentations were performed in a 250 mL Erlenmeyer flask with 50 mL of hydrolysate/flask, with an initial pH of 6, at 25 ± 1 °C, 200 rpm, for 144 h in two sets: (i) wood hydrolysate supplemented with ammonium sulfate, and (ii) wood hydrolysate without an ammonium sulfate supplementation.

2.3. Cell Harvest and Lipid Extraction

The harvest and lipid extraction was carried out according to [21]. Briefly, the cells were harvested by centrifugation and dried at 60 ± 1 °C for 18 h. After that, the cells were treated with a solution of hydrochloric acid for 1 hour and the lipid extraction was performed using a solution of chloroform: methanol (2:1 ratio).

2.4. Analytical Methods

2.4.1. Microbial Growth

The cellular growth was quantified gravimetrically by the weight of the microbial biomass and reported as g/L of the microbial biomass produced (B). Equation (1) was used to calculate the microbial biomass yield ($Y_{B/S}$) and Equation (2) was used to calculate the maximum growth rate (μ_{max}), where X_0 and X_i are the biomass concentration on day t_0 and t_i, respectively.

$$Y_{B/S} = \frac{Total\ biomass\ (g)}{Total\ sugar\ consumed\ (g)} \quad (1)$$

$$\mu_{max} = \frac{X_i - X_0}{t_i - t_0} \quad (2)$$

2.4.2. Sugar Utilization

To quantify the sugar utilization by *R. toruloides*-1588, samples were taken during the fermentation, and the analysis was performed according to [22] using Liquid Chromatography-Mass Spectroscopy with a HILICpak column (5 µm x 150 mm x 4.6 mm) and acetonitrile: water (89:11 v/v) as the mobile phase. The total sugar consumed (S) was reported as g/L and the maximum sugar consumption rate (X_{Smax}) was calculated using Equation (3), where S_i and S_0 are the sugar concentration on day t_i and the first day (t_0), respectively.

$$X_{Smax} = \frac{S_i - S_0}{t_i - t_0} \quad (3)$$

2.4.3. Lipid Content and Fatty Acid Determination

The lipid content and lipid yield ($Y_{L/B}$ and $Y_{L/S}$) were calculated according to Equations (4) and (5), respectively. The determination of the fatty acids was carried out according to [18]. Briefly, the lipids were mixed with methanol and sulfuric acid at 100 ± 1 °C for 20 min. After that, the FAMEs were extracted with hexane and analyzed with Gas Chromatography using (Agilent 7890B, Santa Clara, CA, USA) and an Agilent column (60 m x 250 µm x 0.25 µm). All of the fatty acids were determined using a FAME Mix standard.

$$Y_{L/B} = \frac{Total\ lipids\ (g)}{Total\ biomass\ (g)} \quad (4)$$

$$Y_{L/S} = \frac{Total\ lipids\ (g)}{Total\ sugar\ consumed\ (g)} \quad (5)$$

2.4.4. Total Nitrogen Content

The total nitrogen content (N dissolved + N suspended particles that do not settle) was determined using a Total Organic Carbon Analyzer (Shimadzu-TOC-VCPH, Columbia, SC, USA) with 0.02 mg/L of the detection limit.

2.5. Data Analysis

The data analysis was performed using Origin Lab-Pro® 20.0 (Northampton, MA, USA). The sugar concentration and ammonium sulfate addition effect on the biomass production, sugar utilization, and lipid accumulation were analyzed using a variance analysis with an α = 0.05. All of the tested conditions were run in duplicates.

3. Results

3.1. Substrate Inhibition Analysis

Figure 1 shows the consumption of glucose and xylose by *R. toruloides*-1588. Using C6 wood hydrolysate supplemented with $(NH_4)_2SO_4$ as a substrate, a slow glucose utilization was observed during the first 18 h at initial glucose concentrations of 50 g/L (5.77%), 75 g/L (6.66%), and 100 g/L (20%) in comparison with the tested conditions using a 120 g/L initial glucose concentration with 43.75% of glucose utilization. After that, a high glucose consumption was observed during the next 54 h in all of the tested conditions with an increase in 64.9%, 61.95, 42.5%, and 28.75% for sugar concentrations for 50, 75, 100, and 120 g/L, respectively. The maximum glucose utilization was observed at different fermentation times among the initial glucose concentrations. The highest glucose consumption was observed at 128 h (99.7% consumption) for 50 g/L, 144 h (97.0% and 86.5% consumption) for 75 and 100 g/L, respectively, and 96 h (80% consumption) for 120 g/L. On the contrary, when C6 wood hydrolysate was used without the addition of $(NH_4)_2SO_4$, an increase of 16.29%, 53.0%, 20.0%, and 4.16% in the glucose consumption was observed during the first 18 h with respect to the glucose consumption observed using C6 wood hydrolysate supplemented with $(NH_4)_2SO_4$. The maximum glucose consumption (97%, 81%, and 80%) was observed at 144 h in tested conditions of 75, 100, and 120 g/L of glucose. Conversely, tested conditions with 50 g/L of glucose showed the shortest time (126 h) to achieve the

maximum glucose consumption (>98%) concerning the rest of the tested conditions. The above-stated results show a catabolic repression effect on *R. toruloides*-1588 in both C6 wood hydrolysates when 100 g/L and 120 g/L of sugar were used. For instance, in the tested conditions with the same initial sugar concentrations using C6 hydrolysate with $(NH_4)_2SO_4$, a decrease of 13.4% and 25% in the glucose utilization, respectively, was observed. Moreover, considering the C6 hydrolysate without $(NH_4)_4SO_2$, a decrease of 18.29% and 24% in the glucose utilization was observed at the same concentrations. In both cases, a catabolic repression effect was observed after 96 h of fermentation.

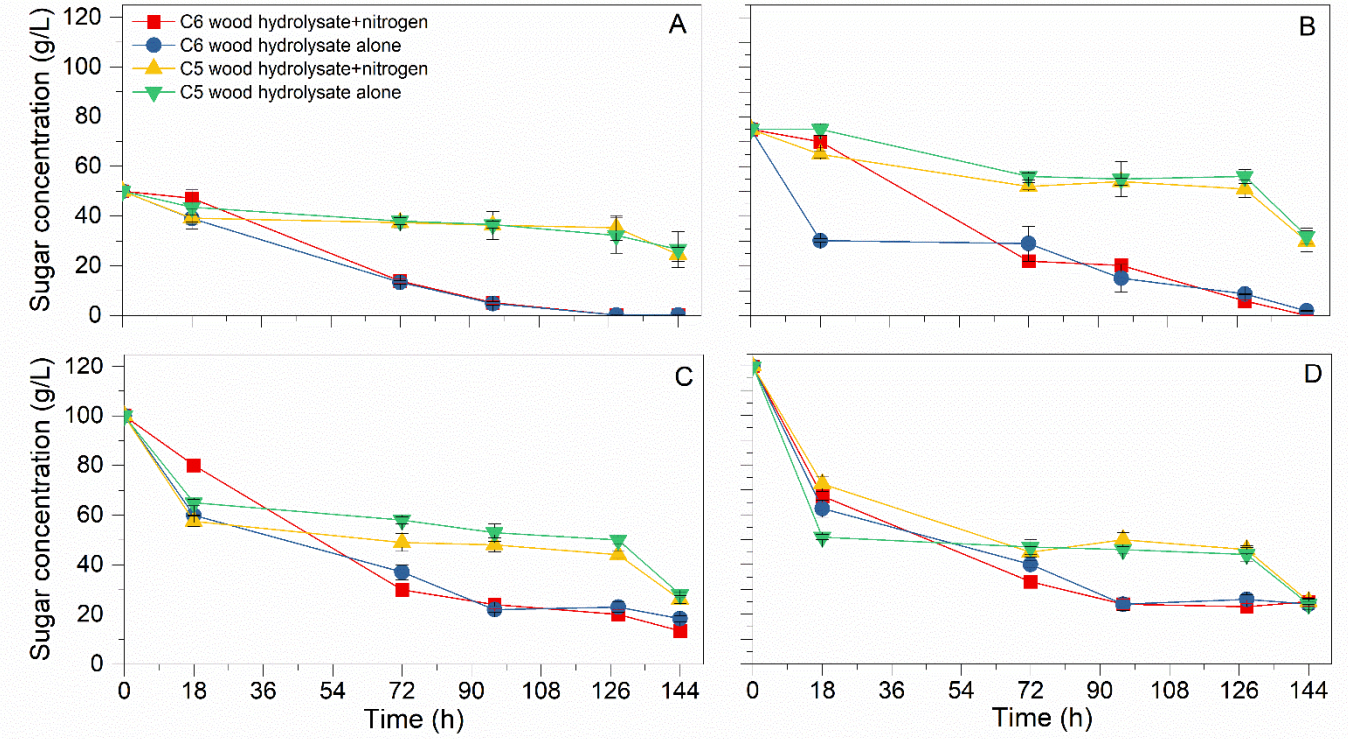

Figure 1. Sugar consumption profile by *R. toruloides*-1588 using wood hydrolysate as a culture media. (**A**) 50 g/L; (**B**) 75 g/L; (**C**) 100 g/L; (**D**) 120 g/L.

The addition of $(NH_4)_2SO_4$ to C5 wood hydrolysate does not show an effect on the sugar utilization due to the total xylose consumption being similar between the tested conditions (Figure 1). For instance, in a treatment with xylose concentrations of 75, 100, and 120 g/L using C5 hydrolysate, a similar xylose consumption of 60%, 73%, and 79% and 57%, 72%, and 80%, was observed in both of the tested conditions, with $(NH_4)_2SO_4$ and without $(NH_4)_2SO_4$, respectively. During the next 108 h of fermentation, the xylose consumption was slow, until a marked increase was observed until the fermentation ended. The results of this study show that *R. toruloides*-1588 encountered a minor inhibition in the xylose utilization compared with the repression caused by glucose using C6 hydrolysate as a growth media. Furthermore, the use and consumption of xylose in this study are promising due to very few studies that have reported the use of xylose as a single carbon in renewable substrates and concentrations higher than 25 g/L [23,24]. Additionally, no literature, to the best of the author's knowledge, has been reported with studies about the inhibition of the substrate using high xylose concentrations.

3.2. Biomass Production Analysis

Figure 2 shows the microbial biomass production by *R. toruloides*-1588. When C6 wood hydrolysate was used in the exponential phase during the first 18 h of cultivation, almost all the tested conditions were observed, except those where 75 g/L of initial sugar concentration using both hydrolysates without a $(NH_4)_2SO_4$ supplementation. Using the

above sugar concentration, almost a constant exponential phase was observed during all fermentation. Likewise, the stationary phase was different for each sugar concentration, for example, in tested conditions with 50 g/L, an increase of 5 g/L in the biomass was observed in C6 hydrolysate supplemented with $(NH_4)_2SO_4$ in comparison with the observed using C6 hydrolysate without a supplementation, with a well-shaded stationary phase and constant exponential phase, respectively. Moreover, when 75 and 100 g/L of sugar were used, a similar tendency was noted in C6 hydrolysate supplemented with $(NH_4)_2SO_4$ with a defined stationary phase of 78 h and for C6 wood hydrolysate without a $(NH_4)_2SO_4$ addition where a constant stationary phase was observed.

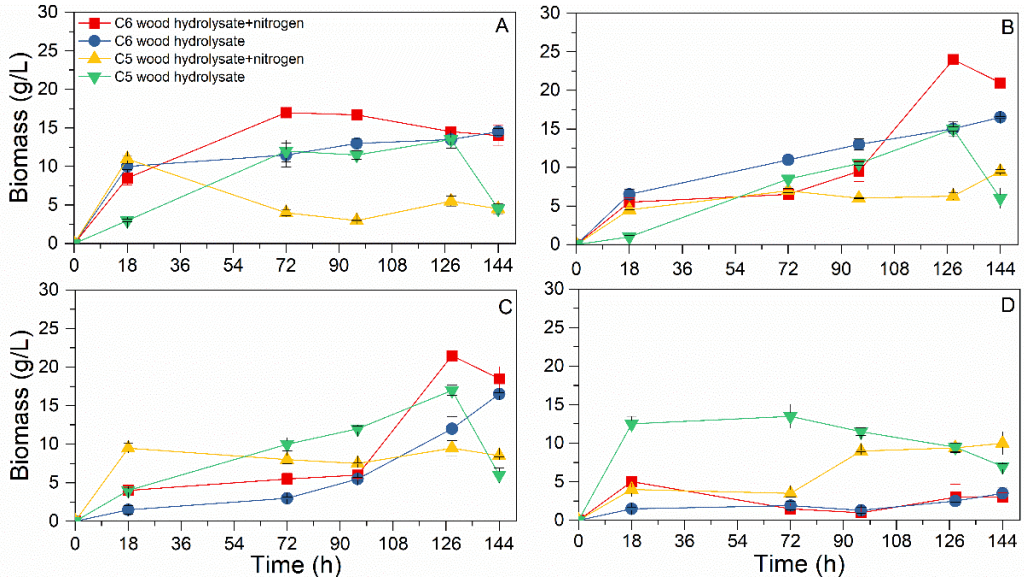

Figure 2. Biomass production by *R. toruloides*-1588 using wood hydrolysate as a culture media. (**A**) 50 g/L; (**B**) 75 g/L; (**C**) 100 g/L; (**D**) 120 g/L.

In tested conditions with C5 hydrolysate, the maximum biomass (17 g/L, 24 g/L, and 21.50 /L) was obtained at 128 h in tested conditions with 50 g/L, 75 g/L, and 100 g/L of sugars, respectively. In the case of C5 hydrolysate without a $(NH_4)_2SO_4$ addition, the maximum microbial biomass of 13.50 g/L was achieved at 72 h with 120 g/L of sugar. The growth profile was different between the initial sugar concentration and hydrolysate supplemented with $(NH_4)_2SO_4$. Furthermore, with sugar concentrations of 50 and 100 g/L, the lag phase was short (less than 6 h, data not shown). In the case of tested conditions with 50 g/L of sugar, an exponential phase of 72 h followed by 56 h of the stationary phase was observed. On the contrary, in the tested conditions with initial sugars of 100 g/L, a constant exponential phase was observed until the 128-hour point was reached. With the growth tendency in the tested conditions with 75 g/L of sugar, a short lag phase of 18 h was noted; after that, an exponential phase of 110 h was observed. In the particular case of the tested conditions with 120 g/L of sugar, a short lag phase (less than 6 h), followed by 18 h of the exponential phase, and 24 h of the stationary phase. The above observations along with the calculated values of the maximum growth rate showed a catabolic repression effect in the microbial growth of ~50% between the two C5 wood hydrolysates used as a culture media in relation to the increase in the initial sugar concentration. Moreover, the results in this study can be used as a baseline to explore the influence of high concentrations of xylose on reducing agents such as the production of NAD+ and NADP in the pentose phosphate pathway, essential compounds during the microbial growth and lipid accumulation [25,26]. Likewise, it has been reported that considerable fluctuations in the pH (3.7 to 8) of the culture media can affect the lipid accumulation more than the microbial growth [27]. Nevertheless, in our previous work, the authors did not observe a significant change in the microbial growth due to the decrease in the pH values (minimum pH value of 5) for

R. toruloides-1588 with a similar substrate and culture conditions (data not shown). Thus, the effect of the pH on the microbial growth has not been investigated in the current study.

In terms of the growth, *R. toruloides*-1588 has a very particular growth pattern which is characterized by a very short lag phase (~6 h) and an exponential phase of ~66 h according to our previous results [17,19–21]. Nevertheless, in this case, the maximum growth at 18 h could be due to a faster growth due to the xylose and nitrogen availability. However, at the same time due to the high concentration of the total nitrogen (~2.5 g/L) and its lowest consumption (~0.3 to 0.5 g/L), the excess of nitrogen can lead to an osmotic shock with an impact on the cell growth. Additionally, another possible hypothesis is that the excess of nitrogen affects the enzymes of the central metabolism of the cells. Furthermore, it has been reported that *R. toruloides* exhibits a short lag phase and a faster increase inthe cellular growth when substrates are rich in xylose and nitrogen [28]. Nonetheless, to have a better knowledge of how this trend is observed in this treatment, detailed proteomic and metabolomic analyses are necessary. Furthermore, another possible reason that affects the microbial growth could be the bioaccumulation of toxic compounds such as furans, organic acids, and phenolic compounds at the end of the fermentation. It is worth pointing out that the substrate (C5 undetoxified wood hydrolysate) used in this study is a complex culture media with an undefined number of inhibitory compounds.

3.3. Nitrogen Effect

It is well known that a lipid accumulation in oleaginous microorganisms is triggered by the limitation of nitrogen sources. Overall, in this study, the total nitrogen consumption was more than 60% but not higher than 90% in the two hydrolysates (Figure 3). Nonetheless, a change in the consumption was observed among the sugar concentrations. For instance, when the yeast was growing in C6 hydrolysate with 50 g/L and 75 g/L of sugar, the total nitrogen consumption was higher (83.3% and 69.3%, respectively) in tested conditions where ammonium sulfate was used as a supplement. Conversely, when 100 g/L and 120 g/L of sugars were used, the highest nitrogen consumption (79.8% and 89.7%, respectively) was noted in conditions where C6 hydrolysate was used alone. In tested conditions where C5 hydrolysate was used, the addition of $(NH_4)_2SO_4$ increases the nitrogen utilization at lower and higher initial sugar concentrations (50 g/L and 120 g/L) with 75.2% and 91.7%, respectively. On the contrary, at 75 g/L and 100 g/L of sugar, a maximum nitrogen consumption of 89.7% and 86.2%, respectively, was observed.

The effect of the addition of $(NH_4)_2SO_4$ on the performance of *R. toruloides*-1588 using both hydrolysates is shown in Table 1. The addition of $(NH_4)_2SO_4$ does not affect the total sugar consumed (p-value = 0.215). In terms of the microbial biomass production using C6 hydrolysate, the addition of $(NH_4)_2SO_4$ promotes a biomass production no matter the initial sugar concentration. Similarly, in tested conditions using C5 hydrolysate, $(NH_4)_2SO_4$ did not show an effect (p-value = 0.256) on the biomass production as the highest values were observed in hydrolysates without a $(NH_4)_2SO_4$ supplementation.

3.4. Lipid Accumulation and Fatty Acid Distribution

The lipid accumulation by *R. toruloides*-1588 using both hydrolysates is shown in Figure 4. Overall, the maximum lipid accumulation was obtained in the tested conditions with C6 hydrolysate. The total lipid accumulation of 8.2 g/L was obtained from 75 g/L of sugar using C6 hydrolysate without ammonium sulfate, followed by tested conditions with 50 g/L and 75 g/L of sugar using C6 hydrolysate with ammonium sulfate where the lipid accumulation was 8 g/L. A minimum lipid accumulation (1.5 g/L) was determined in the tested conditions with 120 g/L of sugar in C6 hydrolysate without a supplementation. Furthermore, a decrease of ~50% in the lipid production was determined in the tested conditions where high initial sugar concentrations were used (100 g/L and 120 g/L). The lipid accumulation in C5 hydrolyzate was significantly lower (p-value = 0.05) compared with that determined in C6 hydrolysate with a maximum value of 3 g/L in the tested conditions with 50 g/L and 120 g/L of sugar using C5 hydrolysate with a $(NH_4)_2SO_4$

supplementation. Similarly, the minimum lipid values were 1.3 g/L and 1 g/L on the same sugar concentrations in C5 hydrolysate without the addition of $(NH_4)_2SO_4$.

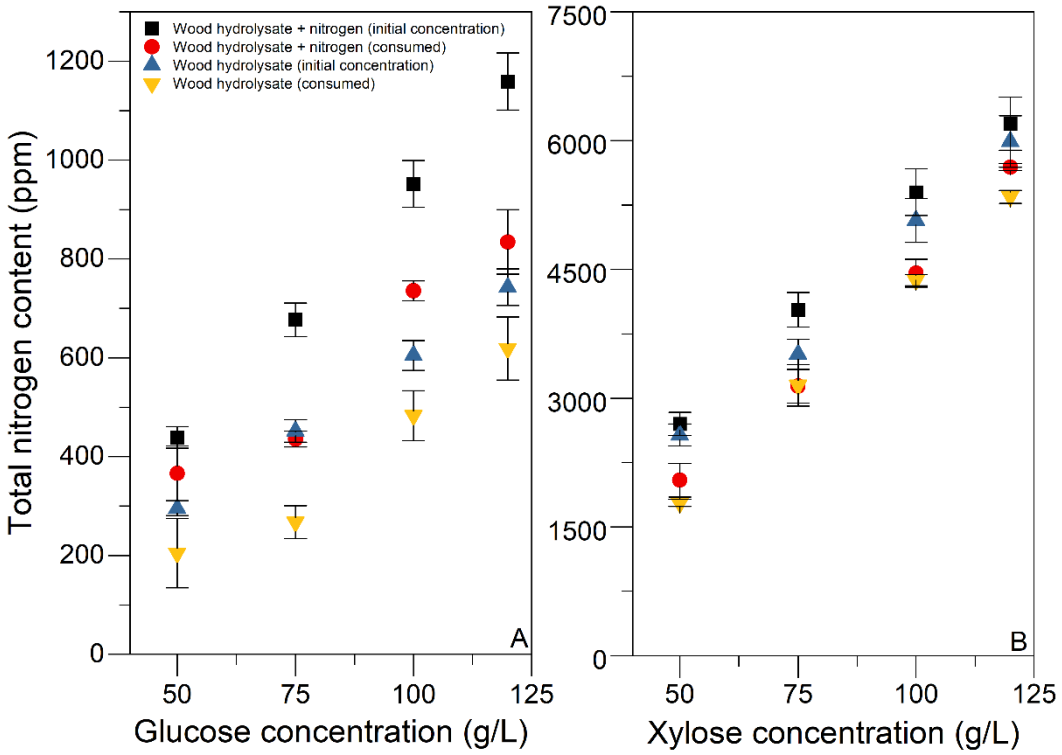

Figure 3. Total nitrogen consumption by *R. toruloides*-1588 using wood hydrolysate as culture media. (**A**) C6 hydrolysate; (**B**) C5 hydrolysate.

Table 1. Performance of *R. toruloides*-1588 using C5 and C6 hydrolysates as a culture media.

Substrate	Initial Sugar (g)	*S* (g/L)	*B* (g/L)	$Y_{B/S}$ (g/g)	μ_{max} (h^{-1})	X_{Smax} (h^{-1})	$Y_{L/S}$ (g/g)
C6 hydrolysate + $(NH_4)_2SO_4$	50	49.90 ± 0.01	17.00 ± 0.42	0.34	0.47	0.50	0.16
C6 hydrolysate		49.90 ± 0.01	14.50 ± 0.45	0.29	0.56	0.61	0.14
C5 hydrolysate + $(NH_4)_2SO_4$		25.43 ± 2.82	11.00 ± 0.32	0.43	0.61	0.60	0.12
C5 hydrolysate		23.46 ± 7.31	13.50 ± 1.13	0.58	0.17	0.88	0.06
C6 hydrolysate + $(NH_4)_2SO_4$	75	74.82 ± 0.07	24.00 ± 0.63	0.32	0.31	0.74	0.11
C6 hydrolysate		73.09 ± 0.13	16.50 ± 0.14	0.23	0.36	2.49	0.11
C5 hydrolysate + $(NH_4)_2SO_4$		45.00 ± 4.24	9.50 ± 0.21	0.21	0.25	0.56	0.06
C5 hydrolysate		43.00 ± 3.25	15.00 ± 0.91	0.35	0.12	1.34	0.03
C6 hydrolysate + $(NH_4)_2SO_4$	100	86.60 ± 1.54	21.50 ± 0.14	0.25	0.22	1.11	0.05
C6 hydrolysate		81.71 ± 1.36	16.50 ± 0.21	0.20	0.11	2.22	0.05
C5 hydrolysate + $(NH_4)_2SO_4$		73.96 ± 1.44	9.50 ± 0.98	0.13	0.53	2.36	0.02
C5 hydrolysate		72.00 ± 1.24	17.00 ± 0.63	0.24	0.22	2.57	0.02
C6 hydrolysate + $(NH_4)_2SO_4$	120	97.00 ± 1.69	5.00 ± 0.14	0.05	0.28	2.92	0.05
C6 hydrolysate		96.00 ± 2.34	3.50 ± 0.77	0.04	0.08	3.19	0.02
C5 hydrolysate + $(NH_4)_2SO_4$		95.00 ± 1.07	10.00 ± 1.48	0.11	0.22	2.64	0.03
C5 hydrolysate		96.00 ± 1.21	13.50 ± 1.54	0.14	0.69	4.00	0.01

The fatty acid profile from lipids produced by *R. toruloides*-1588 using both hydrolysates is shown in Table 2. The saturated fatty acid content was ~1% myristic, palmitic (>25%), and stearic (>15%). In addition, the presence of monounsaturated fatty acids such as oleic acid (>40%) and linoleic (>10%) was observed in both hydrolysates. Finally, the two most abundant polyunsaturated fatty acids were linolenate (>5%) and lignoceric acid (>3%).

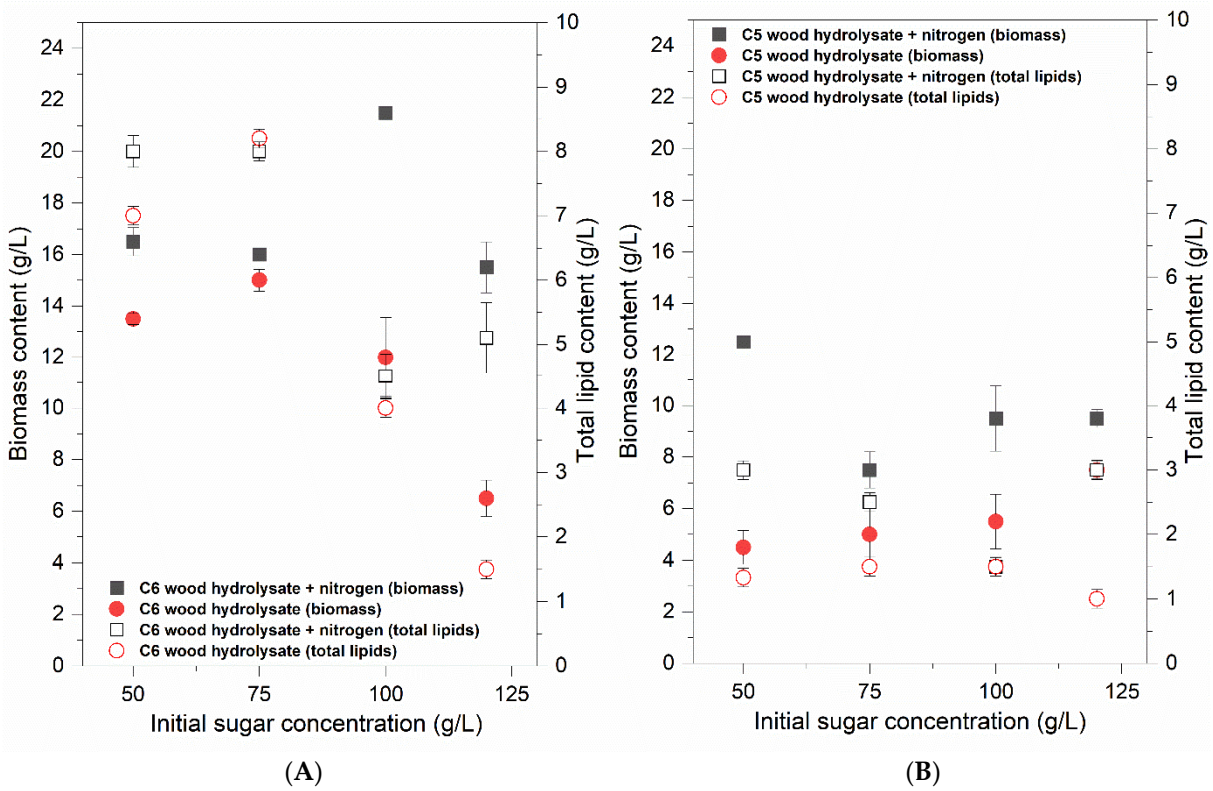

Figure 4. Lipid accumulation profile by *R. toruloides*-1588 using wood hydrolysate as a culture media. (**A**) C6 hydrolysate; (**B**) C5 hydrolysate.

Table 2. Performance of *R. toruloides*-1588 using C5 and C6 hydrolysates as a culture media.

Relative Fatty Acid Content (%)	C6 Wood Hydrolysate + Nitrogen				C6 Wood Hydrolysate			
	Initial Glucose Content (g/L)				Initial Xylose Content (g/L)			
	50	75	100	120	50	75	100	120
Palmitic	23.39 ± 1.16	23.37 ± 1.16	20.15 ± 1.00	21.20 ± 1.06	11.45 ± 0.57	23.06 ± 1.15	22.69 ± 1.13	19.99 ± 0.99
Stearic	14.92 ± 0.74	15.73 ± 0.78	13.09 ± 0.65	18.83 ± 0.94	17.91 ± 0.89	15.53 ± 0.77	11.97 ± 0.59	17.69 ± 0.88
Oleic	39.05 ± 1.95	44.60 ± 2.23	47.48 ± 2.37	34.74 ± 1.73	37.7 ± 1.88	35.33 ± 1.76	41.86 ± 2.09	35.76 ± 1.78
Linoleic	11.30 ± 0.56	6.28 ± 0.31	7.415 ± 0.37	8.31 ± 0.41	15.38 ± 0.76	12.19 ± 0.60	12.33 ± 0.61	8.96 ± 0.44
Linolenate	3.26 ± 0.16	1.92 ± 0.09	3.09 ± 0.15	5.10 ± 0.25	8.40 ± 0.42	5.91 ± 0.29	3.38 ± 0.16	5.41 ± 0.27
Lignoceric	2.19 ± 0.10	2.36 ± 0.11	2.38 ± 0.11	3.88 ± 0.19	1.54 ± 0.07	1.47 ± 0.07	1.79 ± 0.08	3.74 ± 0.18
	C5 wood hydrolysate + nitrogen				C5 wood hydrolysate			
Palmitic	17.50 ± 0.87	17.62 ± 0.88	17.4 ± 0.87	16.49 ± 0.82	16.72 ± 0.83	16.89 ± 0.84	17.47 ± 0.87	17.81 ± 0.89
Stearic	16.73 ± 0.83	18.25 ± 0.91	17.64 ± 0.88	18.17 ± 0.90	17.01 ± 0.85	17.08 ± 0.85	17.81 ± 0.89	19.40 ± 0.97
Oleic	41.49 ± 2.07	41.28 ± 2.06	40.35 ± 2.01	40.18 ± 2.00	41.60 ± 2.08	41.63 ± 2.08	38.8 ± 1.94	38.67 ± 1.93
Linoleic	8.45 ± 0.42	7.345 ± 0.36	8.04 ± 0.40	7.87 ± 0.39	8.28 ± 0.41	8.16 ± 0.40	7.77 ± 0.38	6.96 ± 0.34
Linolenate	5.05 ± 0.25	4.565 ± 0.22	5.15 ± 0.25	4.73 ± 0.23	4.94 ± 0.24	4.99 ± 0.24	4.73 ± 0.23	3.90 ± 0.19
Lignoceric	3.47 ± 0.17	3.345 ± 0.16	3.51 ± 0.17	3.45 ± 0.17	3.77 ± 0.18	3.56 ± 0.17	3.23 ± 0.16	3.70 ± 0.18

4. Discussion

According to the observed results, the increase in the glucose in the C6 wood hydrolysate promotes a partial repression effect. On the contrary, with the increase in the xylose using C5 wood hydrolysate, no catabolic repression effect was observed. In terms of the substrate inhibition, the results in this research are different in comparison to those observed by Li et al. [29] using *R. toruloides* Y4, where they observed that the microbial growth was highly repressed using glucose concentrations between 150 g/L and 300 g/L. It is worth highlighting that in the current study, an undetoxified wood hydrolysate was used as a culture media with $(NH_4)_2SO_4$ as the only supplement, conversely with the minimal media

(Glucose = 10 to 400 g/L, $(NH_4)_2SO_4$ = 12 g/L, KH_2PO_4 = 1 g/L, $MgSO_4 \cdot 7H_2O$ = 1.5 g/L, and yeast extract = 0.5 g/L) used by Li et al. [29]. Thus, the increase in the tolerance to a high glucose concentration could be linked to the availability of the nitrogen source in the culture media that promoted the yeast metabolism as a consequence of the glucose catabolism. Nevertheless, the maximum difference in the xylose consumption (10%) between the substrates was observed when 50 g/L of xylose was used with 48% and 38% of xylose consumption, respectively. Although the trend of the xylose consumption was similar between the two hydrolysates and the four initial sugar concentrations, a faster consumption during the first 18 h was observed in the tested conditions with 100 and 120 g/L of xylose. Likewise, using high xylose concentrations (100 and 120 g/L), an increase of 30% and 45% for the xylose consumption was observed in comparison with the xylose consumed using 50 g/L, and 32.1% and 41.9% in the tested conditions using 75 g/L of xylose. Likewise, the obtained results in this research regarding the biomass production point in the same direction as those observed in the substrate's inhibition. In this study, the maximum biomass production using C6 hydrolysate supplemented with $(NH_4)_2SO_4$ was 21.5 g/L with 100 g/L of glucose. Conversely, Li et al. [29] reported 18.6 g/L using 90 g/L of glucose, showing a decline in the microbial biomass with the increase in the sugar concentration. Similarly, they observed a small decrease in the growth rate with the increase in the sugar from 40 g/L to 150 g/L, and a drastic decrease when the glucose concentrations were higher than 200 g/L. Opposite trends were reported by Fei et al. [30] with a total biomass production of 36.2 g/L using a clarified lignocellulosic hydrolysate with 110 g/L of glucose. Hence, *R. toruloides*-1588 in this study can use glucose more effectively during the biomass production process in comparison with other *R. toruloides* strains.

Even though the results from the sugars' consumption and biomass' production were different from those observed by Li et al. [29] and Fei et al. [30], the lipid accumulation was similar, where a decrease in the lipid production was noted along with the use of sugar concentrations higher than 90 g/L. These results show that the presence of high xylose concentrations in a rich nitrogen media decreases the lipid accumulation and improves the xylose consumption. Nonetheless, the results in this study are promising because *R. toruloides*-1588 accumulates >30% of its dry weight in the form of lipids, a similar accumulation to the theoretical value for this type of strain (~33%). One of the reasons that this can cause a lower lipid accumulation using C5 hydrolysate in comparison with that obtained using C6 hydrolysate is that *R. toruloides* can utilize xylose for the production of sugar alcohols such as D-arabitol, ribitol, or erythritol [28].

In terms of fatty acids, the distribution in this study agrees with the fatty acid profile of *Rhodosporidium* sp. and several vegetable oils [17,23,29–31]. The most significant difference was observed in the produced lipids using C6 wood hydrolysate + $(NH_4)_2SO_4$, showing a two-fold increase in the palmitic and linolenic fatty acids and a slight decrease in the stearic and linoleic fatty acids in the tested conditions with 50 g/L of glucose. Afterward, no significant changes in the fatty acids' distribution and content were observed. On the other hand, the produced lipids using C5 hydrolysate with and without an ammonium sulfate addition do not show major differences in the fatty acids' distribution and content. Overall, the fatty acids' profile observed in the produced lipids is suitable and meets the requirements as a feedstock to produce biodiesel or advanced biofuel.

5. Conclusions

High sugar concentrations using C6 hydrolysate show a proportional catabolic repression effect on *R. toruloides*-1588 with the increase in the glucose. Conversely, *R. toruloides*-1588 does not show catabolic repression using C5 hydrolysate. As expected, the addition of ammonium sulfate as the only supplement promotes a microbial biomass production in the tested conditions using C6 wood hydrolysate and the maximum growth rate in the tested conditions using C5 wood hydrolysate. An important result of this study was that *R. toruloides*-1588 shows a proportional increase in the consumption of xylose (up to 80%) with the increase in the xylose concentration in the culture media. Finally, the use

of undetoxified wood hydrolysates as a culture media is an excellent alternative for the microbial lipid production using *R. toruloides*-1588.

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References

1. Bardi, L. Production of Bio-Oils from Microbial Biomasses. In *Mycoremediation and Environmental Sustainability*; Prasad, R., Ed.; Fungal Biology; Springer International Publishing: Cham, Switzerland, 2018; pp. 61–89, ISBN 978-3-319-77385-8.
2. Chaturvedi, S.; Bhattacharya, A.; Khare, S.K. Trends in Oil Production from Oleaginous Yeast Using Biomass: Biotechnological Potential and Constraints. *Appl. Biochem. Microbiol.* **2018**, *54*, 361–369. [CrossRef]
3. Raud, M.; Kikas, T.; Sippula, O.; Shurpali, N.J. Potentials and Challenges in Lignocellulosic Biofuel Production Technology. *Renew. Sustain. Energy Rev.* **2019**, *111*, 44–56. [CrossRef]
4. Zhu, P.; Abdelaziz, O.Y.; Hulteberg, C.P.; Riisager, A. New Synthetic Approaches to Biofuels from Lignocellulosic Biomass. *Curr. Opin. Green Sustain. Chem.* **2020**, *21*, 16–21. [CrossRef]
5. Osorio-González, C.S.; Gómez-Falcon, N.; Sandoval-Salas, F.; Saini, R.; Brar, S.K.; Ramírez, A.A. Production of Biodiesel from Castor Oil: A Review. *Energies* **2020**, *13*, 2467. [CrossRef]
6. Adrio, J.L. Oleaginous Yeasts: Promising Platforms for the Production of Oleochemicals and Biofuels: Microbial Production of Oleochemicals and Biofuels. *Biotechnol. Bioeng.* **2017**, *114*, 1915–1920. [CrossRef]
7. Osorio-González, C.S.; Hegde, K.; Brar, S.K.; Kermanshahipour, A.; Avalos-Ramírez, A. Challenges in Lipid Production from Lignocellulosic Biomass Using *Rhodosporidium* Sp.; A Look at the Role of Lignocellulosic Inhibitors. *Biofuels Bioprod. Bioref.* **2019**, *13*, 740–759. [CrossRef]
8. Park, Y.-K.; Nicaud, J.-M.; Ledesma-Amaro, R. The Engineering Potential of *Rhodosporidium toruloides* as a Workhorse for Biotechnological Applications. *Trends Biotechnol.* **2018**, *36*, 304–317. [CrossRef]
9. Slininger, P.J.; Dien, B.S.; Kurtzman, C.P.; Moser, B.R.; Bakota, E.L.; Thompson, S.R.; O'Bryan, P.J.; Cotta, M.A.; Balan, V.; Jin, M.; et al. Comparative Lipid Production by Oleaginous Yeasts in Hydrolyzates of Lignocellulosic Biomass and Process Strategy for High Titers: High Titer Lipid Production by Yeasts in Lignocellulosic Hydrolyzates. *Biotechnol. Bioeng.* **2016**, *113*, 1676–1690. [CrossRef]
10. Tkáčová, J.; Klempová, T.; Čertík, M. Kinetic Study of Growth, Lipid and Carotenoid Formation in β-Carotene Producing Rhodotorula Glutinis. *Chem. Pap.* **2018**, *72*, 1193–1203. [CrossRef]
11. Nair, A.S.; Sivakumar, N. Enhanced Production of Biodiesel by *Rhodosporidium toruloides* Using Waste Office Paper Hydrolysate as Feedstock: Optimization and Characterization. *Fuel* **2022**, *327*, 125174. [CrossRef]
12. Tuhanioglu, A.; Hamamci, H.; Alpas, H.; Cekmecelioglu, D. Valorization of Apple Pomace Via Single Cell Oil Production Using Oleaginous Yeast *Rhodosporidium toruloides*. *Waste Biomass Valor.* **2022**, *8*, 1–15. [CrossRef]
13. Vorapreeda, T.; Thammarongtham, C.; Cheevadhanarak, S.; Laoteng, K. Alternative Routes of Acetyl-CoA Synthesis Identified by Comparative Genomic Analysis: Involvement in the Lipid Production of Oleaginous Yeast and Fungi. *Microbiology* **2012**, *158*, 217–228. [CrossRef]
14. Coradetti, S.T.; Pinel, D.; Geiselman, G.M.; Ito, M.; Mondo, S.J.; Reilly, M.C.; Cheng, Y.-F.; Bauer, S.; Grigoriev, I.V.; Gladden, J.M.; et al. Functional Genomics of Lipid Metabolism in the Oleaginous Yeast *Rhodosporidium toruloides*. *eLife* **2018**, *7*, e32110. [CrossRef]
15. Amaretti, A.; Raimondi, S.; Sala, M.; Roncaglia, L.; De Lucia, M.; Leonardi, A.; Rossi, M. Single Cell Oils of the Cold-Adapted Oleaginous Yeast Rhodotorula Glacialis DBVPG 4785. *Microb. Cell Fact.* **2010**, *9*, 73. [CrossRef] [PubMed]
16. Dourou, M.; Aggeli, D.; Papanikolaou, S.; Aggelis, G. Critical Steps in Carbon Metabolism Affecting Lipid Accumulation and Their Regulation in Oleaginous Microorganisms. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2509–2523. [CrossRef]

17. Osorio-González, C.S.; Hegde, K.; Ferreira, P.; Brar, S.K.; Kermanshahipour, A.; Soccol, C.R.; Avalos-Ramírez, A. Lipid Production in *Rhodosporidium toruloides* Using C-6 and C-5 Wood Hydrolysate: A Comparative Study. *Biomass Bioenergy* **2019**, *130*, 105355. [CrossRef]
18. Osorio-González, C.S.; Saini, R.; Hegde, K.; Brar, S.K.; Lefebvre, A.; Avalos-Ramírez, A. Inhibitor Degradation by *Rhodosporidium toruloides* NRRL 1588 Using Undetoxified Wood Hydrolysate as a Culture Media. *Biomass Bioenergy* **2022**, *160*, 106419. [CrossRef]
19. Osorio-González, C.S.; Saini, R.; Hegde, K.; Brar, S.K.; Avalos Ramirez, A. Furfural Degradation and Its Effect on *Rhodosporidium toruloides*-1588 during Microbial Growth and Lipid Accumulation. *Bioresour. Technol.* **2022**, *359*, 127496. [CrossRef]
20. Saini, R.; Osorio-Gonzalez, C.S.; Hegde, K.; Brar, S.K.; Vezina, P. Effect of Creating a Fed-Batch like Condition Using Carbon to Nitrogen Ratios on Lipid Accumulation in *Rhodosporidium toruloides*-1588. *Bioresour. Technol.* **2021**, *337*, 125354. [CrossRef]
21. Saini, R.; Osorio-Gonzalez, C.S.; Hegde, K.; Kaur Brar, S.; Vezina, P. A Co-Fermentation Strategy with Wood Hydrolysate and Crude Glycerol to Enhance the Lipid Accumulation in *Rhodosporidium toruloides*-1588. *Bioresour. Technol.* **2022**, *364*, 127821. [CrossRef]
22. Saini, R.; Hegde, K.; Osorio-Gonzalez, C.S.; Brar, S.K.; Vezina, P. Evaluating the Potential of *Rhodosporidium toruloides*-1588 for High Lipid Production Using Undetoxified Wood Hydrolysate as a Carbon Source. *Energies* **2020**, *13*, 5960. [CrossRef]
23. Lopes, H.J.S.; Bonturi, N.; Kerkhoven, E.J.; Miranda, E.A.; Lahtvee, P.-J. C/N Ratio and Carbon Source-Dependent Lipid Production Profiling in Rhodotorula Toruloides. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 2639–2649. [CrossRef]
24. Lopes, H.J.S.; Bonturi, N.; Miranda, E.A. Induction of Resistance Mechanisms in Rhodotorula Toruloides for Growth in Sugarcane Hydrolysate with High Inhibitor Content. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 9261–9272. [CrossRef]
25. Tiukova, I.A.; Brandenburg, J.; Blomqvist, J.; Sampels, S.; Mikkelsen, N.; Skaugen, M.; Arntzen, M.Ø.; Nielsen, J.; Sandgren, M.; Kerkhoven, E.J. Proteome Analysis of Xylose Metabolism in Rhodotorula Toruloides during Lipid Production. *Biotechnol. Biofuels* **2019**, *12*, 137. [CrossRef]
26. Tiukova, I.A.; Prigent, S.; Nielsen, J.; Sandgren, M.; Kerkhoven, E.J. Genome-scale Model of *Rhodotorula Toruloides* Metabolism. *Biotechnol. Bioeng.* **2019**, *116*, 3396–3408. [CrossRef]
27. Ling, J.; Nip, S.; Shim, H. Enhancement of Lipid Productivity of *Rhodosporidium toruloides* in Distillery Wastewater by Increasing Cell Density. *Bioresour. Technol.* **2013**, *146*, 301–309. [CrossRef]
28. Jagtap, S.S.; Rao, C.V. Production of D-Arabitol from d-Xylose by the Oleaginous Yeast *Rhodosporidium toruloides* IFO0880. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 143–151. [CrossRef]
29. Li, Y.; Zhao, Z.; Bai, F. High-Density Cultivation of Oleaginous Yeast *Rhodosporidium toruloides* Y4 in Fed-Batch Culture. *Enzym. Microb. Technol.* **2007**, *41*, 312–317. [CrossRef]
30. Fei, Q.; O'Brien, M.; Nelson, R.; Chen, X.; Lowell, A.; Dowe, N. Enhanced Lipid Production by *Rhodosporidium toruloides* Using Different Fed-Batch Feeding Strategies with Lignocellulosic Hydrolysate as the Sole Carbon Source. *Biotechnol. Biofuels* **2016**, *9*, 130. [CrossRef]
31. Maza, D.D.; Viñarta, S.C.; Su, Y.; Guillamón, J.M.; Aybar, M.J. Growth and Lipid Production of Rhodotorula Glutinis R4, in Comparison to Other Oleaginous Yeasts. *J. Biotechnol.* **2020**, *310*, 21–31. [CrossRef]