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Evaluating the Potential of *Rhodosporidium toruloides*-1588 for High Lipid Production Using Undetoxified Wood Hydrolysate as a Carbon Source

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Abstract: The study aims to explore microbial lipid production using an abundant and low-cost lignocellulosic biomass derived from forestry residues. Sugar-rich undetoxified hydrolysate was prepared using hardwood and softwood sawdust and used for lipid production as a carbon source from an oleaginous yeast, *Rhodosporidium toruloides*-1588. The maximum biomass obtained was 17.09 and 19.56 g/L in hardwood and softwood hydrolysate, respectively. Sugar consumption in both hydrolysates was >95%, with a maximum lipid accumulation of 36.68% at 104 h and 35.24% at 96 h. Moreover, *R. toruloides*-1588 exhibited tolerance to several toxic compounds such as phenols, organic acids and furans present in hydrolysates. The lipid characterization showed several monosaturated and polyunsaturated fatty acids, making it a potential feedstock for biofuels and oleochemicals production. This study confirms the credibility of *R. toruloides*-1588 as a suitable lipid producer using hydrolysates from forestry residues as a substrate. Additionally, lipids obtained from *R. toruloides*-1588 could be a potential feedstock for advanced biofuel production as well as for food and pharmaceutical applications.

Keywords: *Rhodosporidium toruloides*; microbial lipid; lignocellulosic biomass; forestry residue; undetoxified hydrolysate

1. Introduction

A microorganism tends to accumulate lipids in the form of triglycerides, under the nutrient stress conditions, either during nitrogen limitation or excess of carbon [1]. Microorganisms that can store more than 25% lipid (*w/w*) of their total dry cell weight (DCW) are known as oleaginous strains. For instance, *Lipomyces starkeyi*, *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Cryptococcus curvatus*, among others. Their capability to thrive on a wide variety of inexpensive substrates renders them an outstanding alternative to conventional oil sources [2]. Microbial lipids have applications in several industries including biofuels, food, pharmaceutical, and chemical industries. For example, docosahexaenoic acid and eicosapentaenoic acid provide aid in the cardiovascular system, eye functioning, and hormonal balance. Similarly, gamma-linolenic acid is known to have anti-cancer properties. Lipids can be transformed into a broad range of biofuels through biochemical or chemical conversions such as biodiesel (mono-alkyl esters), fatty alkanes, and fatty aldehydes [3]. The critical challenges in lipid production are the availability of substrates, process cost, and that they should comply with food security. Over the past decades, biorefinery has moved towards the usage of lignocellulosic-based biomass as an alternative substrate for microbial-based lipid production [2].

Lignocellulosic biomass is known as an inexpensive and abundant source of carbon. It generally requires a pretreatment to interrupt the linkage between lignin, hemicellulose, and cellulose, followed by the action of cellulolytic enzymes to convert polymeric sugars into monomeric form. Sources of lignocellulosic biomass include but are not limited to the forestry, agricultural, municipal sectors. Canada possesses 10% of the world's forests and represents 58% of total feedstock, while annual agriculture and municipal feedstock account for 39% and 3%, respectively [4]. Hence, this could be a renewable option to utilize sugar-rich hydrolysate derived from forestry biomass to produce microbial lipids.

Over the past decades, a huge number of strains have been exploited for lipid production using biomass hydrolysate. However, the foremost challenge is the consumption of sugars in the presence of inhibitors that generally release into hydrolysate after the lignocellulose pretreatment. These compounds generally hinder the use of substrates by interfering in the metabolic pathways of microorganisms [5]. Hence, it is very important to use a robust strain that should be capable of consuming a wide variety of carbon sources as well as have high inhibitor tolerances. A recent study has shown that random mutagenesis or genetic engineering could lead to an increase in lipid production, carbon consumption, or inhibitor tolerance. For instance, Guo et al. [6] described the surge in the lipid production by 43.6% in *Rhodosporidium toruloides* AS 2.1389 when the strain was irradiated with ultraviolet rays.

R. toruloides, a basidiomycetes yeast, has been widely studied over the past years and reported to co-metabolize pentose and hexose sugars. *R. toruloides* was found to yield 100 g/L biomass and accumulate 50–70% lipid of its total DCW [5]. It is also known as red yeast because of the production of carotenoids, such as astaxanthin, β-carotene, and torularhodin. These are known as high-value compounds for the pharmaceutical and food industries [2]. Consequently, research on *R. toruloides* strain has been carried out to gather knowledge on multiple aspects of its genome. Nevertheless, the research on *R. toruloides* has begun to exploit the fields such as inhibitor tolerance, genetic and metabolic engineering, growth and lipid production behavior in various carbon sources such as sucrose, xylose, crude glycerol, and wood hydrolysates [2,7].

The present study aims to evaluate the lipid production ability of *Rhodosporidium toruloides*-1588 in undetoxified hydrolysate derived from forestry biomass. Its lipid production ability, fatty acids methyl esters (FAMEs), biomass, sugar consumption, and inhibitory effect were studied and evaluated. In addition, the effect of the addition of nitrogen source on *R. toruloides* growth, lipid titer and different types of polyunsaturated fatty acids were also analyzed.

2. Materials and Methods

2.1. Enzyme Production and Extraction

Aspergillus niger (NRRL-2001) was acquired from the Agricultural Research Service (NRRL) Culture Collection (USA). *A. niger* was maintained on potato dextrose agar (PDA) plate and incubated at 30 °C until black spores appeared. Spores were then harvested, and spore count was determined with a hemocytometer followed by aliquot preparation of spores in 1% of Tween-80 and stored at −20 °C. *A. niger* was cultivated in apple pomace (AP) supplemented with 1% rice husk at 75% moisture content, as performed by Dhillon et al. [8].

2.2. Enzyme Activity

One gram of fermented sample was collected at the end of fermentation and dissolved in 15 mL of 50 mM citrate buffer (pH 4.8). The solution was kept in a shaking condition for 30 min at 200 rpm, followed by separation of enzyme solution from biomass using a centrifuge at $9000\times g$, 4 °C for 15 min (Sorvall RC-5 Plus) followed by the supernatant analysis of enzyme activity.

The total activity of cellulase was measured using the standard filter paper assay method. Briefly, the reaction mixture for filter paper assay contained 500 μL enzyme solution, 1 mL citrate buffer

(50 mM and pH 4.8) and the filter paper strip of Whatman no.1. The two different enzyme dilutions were prepared, i.e., one dilution should release a little less and the other dilution a little more than 2.0 mg of glucose. The samples were incubated for 60 min at 50 °C followed by the addition of 3.0 mL of 3,5-dinitrosalicylic acid (DNS) in solution. The reaction mixture was boiled for 10 min and then cooled down by keeping tubes in ice. The amount of sugar release was calculated using a UV-visible spectrophotometer (Cary-50, Varian Inc., Palo Alto, CA, USA) at 540 nm.

2.3. Pretreatment and Enzymatic Saccharification

Hardwood (Maple wood) and softwood (Fir wood) sawdusts were obtained from Quebec's Forestry Industry Council. Biomass was dried at 60 ± 1 °C for 12 h. Sawdust was further milled using a planetary ball mill (Retsch Corporation; PM100) at 500 rpm for 15 min. The resulting milled wood biomass was separated using a sieve with a final size of 300 μm - 1 mm [9]. Afterward, the wood sawdust was used for the pretreatment and enzymatic saccharification.

Both types of sawdusts were pretreated using dilute alkali (1% NaOH). The solid-to-liquid ratio was kept at 1:10 (*w/v*). The slurry was then autoclaved at 121 °C/15 psi for 30 min. Afterward, the slurry was centrifuged (7000× *g*/15 min) and the solid fraction was washed using tap water and dried at 45 ± 1 °C for 30 min. The enzymatic saccharification was performed in a Multitron® shaker at 40 ± 1 °C for 24 h with a crude enzyme extract loading rate of 12 IU/gram of dry substrate with a substrate loading rate of 10% (*w/v*) with respect to the volume of slurry at pH 4.8, using a citrate buffer.

2.4. Microorganism Maintenance and Inoculum Preparation Conditions

R. toruloides-1588 was obtained from the NRRL Culture Collection of the USA. *R. toruloides*-1588 was maintained in Yeast Malt (YM) agar plates as described by Osorio-Gonzalez et al. [7]. Yeast was pre-cultured till three generations in YM media, i.e., yeast extract and malt extract (3.0 g/L each), peptone (5.0 g/L), and glucose (10.0 g/L). This media composition was also used as a synthetic media in further experiments [7]. The inoculum was prepared in three different steps with incubation conditions of 25 °C, 180 rpm for 18 h: (1) a 2 mL YM broth was inoculated with a single colony picked from a fresh plate and incubated; (2) Inoculum was then transferred (1:100 *v/v*) to 5 mL YM broth and incubated; (3) Final inoculum was transferred (1:100 *v/v*) to 50 mL YM broth and incubated at 25 °C and 180 rpm.

2.5. Lipid Production

For the lipid production, yeast was cultured in 50 mL of undetoxified hardwood and softwood hydrolysate. Two sets of the experiment were run, one with the addition of 1 g/L ammonium sulfate and another without any addition, and the pH was adjusted to 6.0. The hydrolysates were inoculated using third-generation inoculum with an optical density (OD) at 600 nm of 0.3 and incubated at 25 °C and 180 rpm. The samples were collected at a regular interval of time throughout the fermentation process. All the experiments were performed in duplicates. The lipid yield, content and concentration were evaluated using Ma et al. [10] equations as follows:

$$\text{Lipid Content} = (\text{Lipid weight (g)})/(\text{Biomass weight (g)}) \tag{1}$$

$$\text{Lipid Concentration} = (\text{Lipid weight (g)})/(\text{Culture Volume (L)}) \tag{2}$$

$$\text{Lipid accumulation (\%)} = (\text{Lipid concentration (g/L)}/\text{Biomass concentration (g/L)}) \times 100 \tag{3}$$

2.6. Lipid Extraction

Lipid extraction was conducted, as performed by Osorio-Gonzalez et al. [7]. Briefly, cells were separated from the fermented broth using centrifugation (15,000× *g* for 3 min), and dried at 60 °C until constant dry weight. The cell lysis was performed by boiling them in the presence of 1 M/L HCl for 1 h followed by 3.75 mL chloroform:methanol ratio (2:1 *v/v*) addition in the samples. The mixtures were

then mixed for 15 min using a vortex mixer and 1.25 mL of chloroform was added, then the sample was vortexed again for 1 min; lastly, 1.25 mL of 1 M NaCl was added to the mixture and centrifuged ($7000\times g$ for 15 min).

2.7. Analytical Methods

2.7.1. Cell Growth Analysis

Cell growth was measured in grams of DCW. The yeast cells were separated from the fermented broth using centrifugation ($15,000\times g$ for 2 min), followed by cell washing with phosphate-buffered saline (PBS, pH–7.0), and drying at 60 °C until a constant weight was achieved and expressed in g L^{-1}.

2.7.2. Inhibitors and Sugar Analysis

Sugars and inhibitors analysis was performed, as explained by Osorio-Gonzalez et al. [7]. Briefly, total reducing sugar was analyzed using the DNS method, followed by sugar quantification in UV-visible spectrophotometer (Cary-50, Varian Inc., Palo Alto, CA, USA) at 540 nm. The composition of sugar and inhibitors was quantified using a liquid chromatography-mass spectrophotometer (Thermo Scientific Liquid TSQ Quantum Access Mass Spectrometer) [7]. All compounds were quantified and determined using standards obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.7.3. Fatty Acid Determination (FAME)

The FAMEs were quantified using the conditions reported by Osorio-Gonzalez et al. [7]. Crude lipid was esterified in sulfuric acid and methanol at 100 °C for 20 min. Later, the samples were cooled at room temperature and FAMEs were extracted using hexane. FAME composition was quantified using gas chromatography (Agilent 7890B), with the specification provided by Osorio-Gonzalez et al. [7].

The cetane number (CN) was calculated using Equation (4) provided by Fei et al. [11].

$$CN = 62.2 + 0.0074X_a + 0.115X_b + 0.177X_c - 0.103X_d - 0.279X_e - 0.366X_f \qquad (4)$$

where values of X are the weight percentage of fatty acids such X_a is of methyl myristate; X_b is of palmitic acid; X_c is of stearic acid; X_d is of oleic acid; X_e is of linoleic acid; and X_f is of linolenic acid.

2.7.4. Statistical Analysis

The statistical analysis of the data was conducted using Minitab® 17.1.0 (Minitab Inc., State College, PA, USA). Parameter effects such as biomass production and lipid accumulation were evaluated by one-way ANOVA (Analysis of Variance). Statistical significance in the mean values of various measured parameters was calculated and compared with the Fisher test (95% level of confidence).

3. Results and Discussion

### 3.1. Wood Hydrolysate and Its Compositions

The hydrolysate was prepared from sawdust residues of the Maple tree (hardwood) and Fir tree (softwood). The total reducing sugar of hardwood and softwood hydrolysate was obtained as 46.01 and 45.25 g/L after alkaline pretreatment and enzymatic saccharification (12 International enzymes units/gram of dry substrate). The sugars and inhibitors compositions are shown in Table 1. The pretreatment of biomass was performed using dilute alkali (1% Sodium hydroxide). The presence of low amounts of organic acids, phenolic compounds, and furfurals in hydrolysates could be because alkali solution is known to dissolve 60–80% of lignin and phenolic compounds [12].

Table 1. Sugars and inhibitors composition detected in hardwood and softwood hydrolysates.

Components	Hardwood Hydrolysate (g/L)	Softwood Hydrolysate (g/L)
Glucose	12.64 ± 1.52	13.27 ± 1.23
Xylose	10.16 ± 0.95	13 ± 0.89
Galactose	12.96 ± 0.81	13.66 ± 1.12
Fructose	3.24 ± 0.21	3.30 ± 0.37
Trehalose	0.03 ± 0.00	2.4 ± 0.07
Furfural	0.067 ± 0.01	0.057 ± 0.01
5-hydroxymehtyl furfural	0.025 ± 0.002	0.035 ± 0.001
Vanillic acid	0.034 ± 0.003	0.042 ± 0.001
Vanillin	0.007 ± 0.0005	0.01 ± 0.002
Levulinic acid	0.007 ± 0.0003	0.007 ± 0.0006

3.2. Biomass Growth and Sugar Consumption in the Wood Hydrolysates

Biomass growth profile in synthetic media (SM), hardwood (HW), and softwood (SW) hydrolysates are presented in Figure 1. The cells continued to be in exponential phase until 72 h followed by a stationary phase in hardwood hydrolysate while the two exponential phases (i.e., 18 h and 72 h) were observed in softwood hydrolysate. In general, microorganisms demonstrated diauxic growth when subjected to two different carbon sources in the culture media. Specifically in *Rhodosporidium* sp., once the glucose was consumed from the media it causes the yeast to consume another available carbon source resulting in the second exponential phase for a shorter duration [7]. The maximum biomass observed was 5.2 and 6 g/L in hardwood and softwood, respectively. In synthetic media, cells continue to be in the exponential phase until 72 h followed by the stationary phase. *R. toruloides*-1588 reached maximum biomass of 9 g/L in synthetic media.
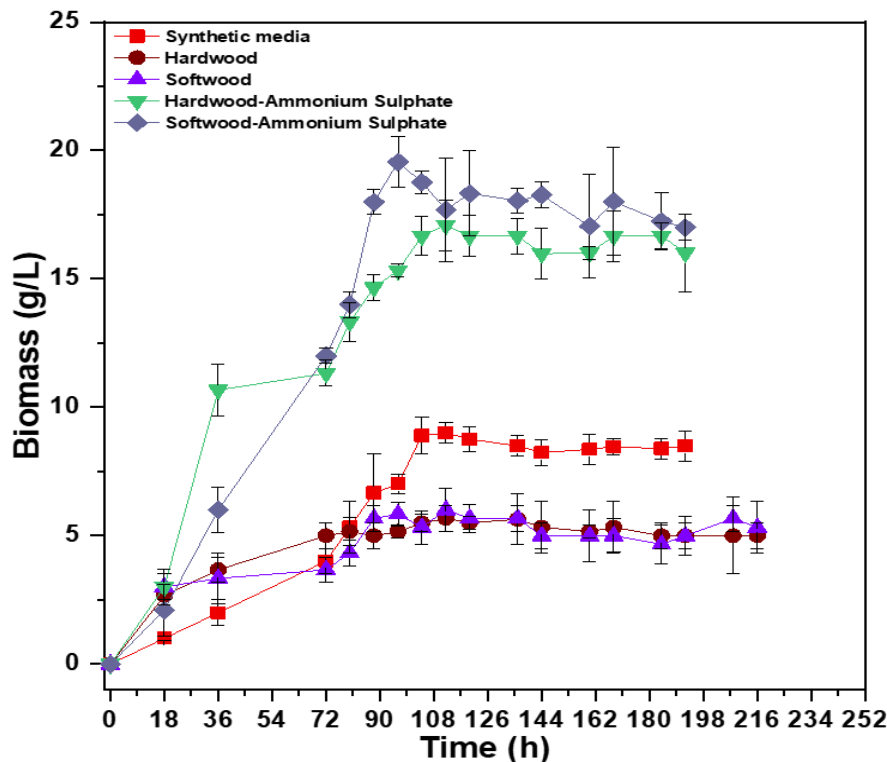
Figure 1. Growth profile of Rhodosporidium toruloides-1588 in different media.

Another possible reason for lower biomass could be a very high C/N (carbon to nitrogen) ratio or a very low amount of available nitrogen in the hydrolysate. This is because the carbon causes

osmotic shock when present in large amounts in the media due to a decrease in Krebs cycle activity, reduced energy production, and intracellular increase of critic acid [13]. Total nitrogen in hardwood and softwood hydrolysate was observed to be 85 and 77 mg/L, respectively, and it remained unused throughout the batch experiment (C/N > 200). It has already been established that lipid accumulation occurs at C/N ratio 20 and which continue to vary till C/N ratio 120 [2,7]. However, at a very high C/N ratio, lipid and biomass accumulation tends to decrease too [3,14]. Hence, another experiment was performed with the supplementation of ammonium sulfate (1 g/L) in both the hydrolysates. Ammonium sulfate has 0.21% nitrogen content in it [15] and it resulted in a change of C/N ratio to 87.6. The addition of ammonium sulfate has not only affected biomass growth but also led to near-complete consumption of sugars (>95%) in both hydrolysates. This could be due to the availability and use of nitrogen sources at the beginning of fermentation, which allowed the yeast to utilize the sugar in the hydrolysate. Nitrogen is a known activator of isocitrate dehydrogenase (ICDH). In general, ICDH catalyzes the isocitrate conversion into α-ketoglutarate in the Krebs cycle and releases the nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) as a by-product, which further takes part in the electron transport chain for energy production and microbial reproduction. On the other hand, the limitation of nitrogen results in the inactivation of ICDH which further leads to citrate accumulation in mitochondria and ultimately converts into lipids through a series of conversions [16]. The maximum biomass achieved was 17.09 and 19.56 g/L in hardwood and softwood hydrolysates, respectively (Figure 1). This result is promising as the yeast was able to thrive in undetoxified wood hydrolysate without any supplementation of micronutrients such as zinc sulfate, magnesium sulfate, ferric chloride, and manganese sulfate, which can be seen in several published articles [17–20]. Lower biomass in the hydrolysate could be due to the presence of inhibitors in the hydrolysates. Even the effect of inhibitors can also be seen on the sugar consumption profile of *R. toruloides*.

On the other hand, in hydrolysate, 26% and 31% consumption of sugars were observed in hardwood and softwood, respectively, while 98% of the sugar has been consumed in control. Synthetic media has only glucose as a carbon source hence resulted in rapid consumption of glucose within 72 h of fermentation, while hydrolysate has both hexose, pentose and inhibitors which might have hindered the sugar consumption as well as biomass growth. In addition, more than 70–80% of glucose consumption was seen while only ~10% and ~14% of consumption were seen in the case of xylose in hardwood and softwood hydrolysates, respectively, as shown in Figure 2. This could be the reason for the double exponential phase of *R. toruloides*-1588 in softwood hydrolysate (Figure 1). This result states the difference in consumption of C6 and C5 sugars are due to metabolic limitation. Similarly, when *R. toruloides*-1588 were grown in C5 and C6 hydrolysate separately, <50% and >90% of xylose and glucose consumption were seen, respectively [7]. The diauxic growth can also be seen in other blends of carbon sources such as glucose and glycerol. For instance, Bommareddy et al. [21] cultivated the *R. toruloides* in mixed media of glucose (10 g/L) and glycerol (40 g/L). The authors observed the consumption of glycerol (~37%) only after the glucose was consumed (>90%) resulted in a double exponential phase at 7 h and 30 h. On the other hand, the difference in sugar consumption in hardwood and softwood hydrolysate could be due to the differences in furfural concentrations, i.e., 57 mg/L in softwood, while 66 mg/L in hardwood. Glucose can still be consumed by yeast but increasing the furfural concentration has affected xylose consumption. Based on the above results, the lower the furfural concentration, the higher will be the sugar consumption. Similarly, Zhao et al. [18] demonstrated that the increase in furfural concentration to 1.0 g/L, glucose consumption was seen while xylose utilization by *R. toruloides* AS 2.1389 was inhibited, hence leading to 60% lower biomass production. Nevertheless, to further increase the C5 sugar consumption in *R. toruloides* sp., several processes and engineering methodologies have been developed, for instance, phenotypic heterogeneity, epigenetic inheritance, evolutionary engineering, random mutagenesis, and site-directed mutagenesis [7]. So far, to improve the *Rhodosporidium* strains, different engineering strategies are being employed by overexpressing enzymes such as malic acid, acetyl-CoA citrate, ATP-citrate lyase,

and diglyceride acyltransferase. Nevertheless, research and literature on pentoses consumption in *Rhodosporidium* sp. is limited or null, hence further studies and strategies are required to further increase the sugar consumption as well as lipid accumulation.
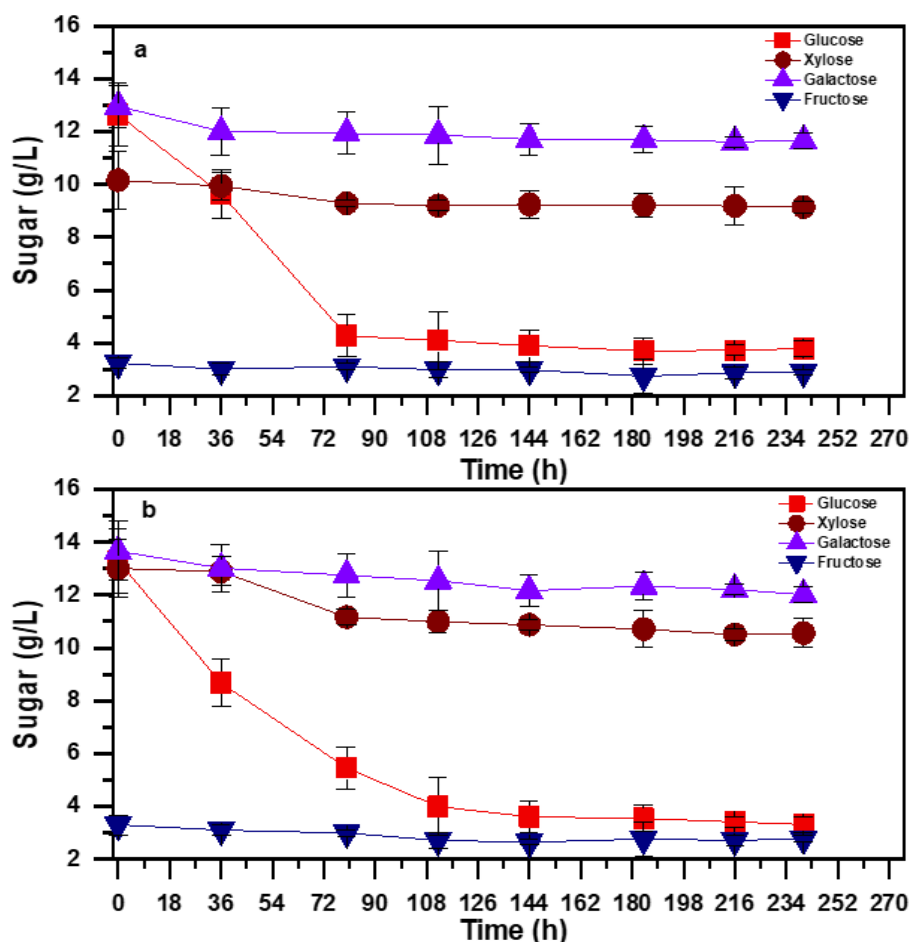

Figure 2. Sugar reduction profile during the yeast fermentation: (**a**) consumption profile in hardwood hydrolysate; (**b**) consumption profile in softwood hydrolysate.

3.3. Lipid accumulation and FAME Composition

The lipid accumulation in hydrolysates (hardwood and softwood) without ammonium sulphate addition was 0.25 g lipid/g biomass, and 0.22 g lipid/g biomass, respectively. This lipid accumulation was lower than obtained using synthetic media (0.27 g lipid/g biomass). Nevertheless, we observed a different time to reach the maximum lipid accumulation (104 h, 96 h, and 118 h, respectively), which from the process point of view is a promising result, as the short production process required less energy. The above fact can contribute to increasing the profitability of the entire process because in both the hydrolysates, the maximum lipid accumulation time was earlier than in the control.

On the other hand, the addition of ammonium sulfate (1 g/L) had a significant positive effect (p-value ≤ 0.05) on the lipid accumulation in *R. toruloides*-1588 and resulted in ~30% increase in lipid content, i.e., 0.37 g lipid/g biomass and 0.35 g lipid/g biomass accumulation in hardwood and softwood hydrolysate, respectively. In general, under stress conditions, *Rhodosporidium toruloides* tend to accumulate lipids in the early stages of its growth [7]. The addition of ammonium sulfate as a nitrogen source in the hydrolysate increased lipid accumulation (Supplementary Figure S1). There is no denying the fact that nutrient imbalance in media triggers lipid accumulation in oleaginous yeast. When key nutrients such as nitrogen run out, cells cannot multiply and excess of carbon is then converted into storage lipids. Therefore, the initial C/N ratio in media has a key role in lipid accumulation. For

instance, Papanikolaou et al. [22] reported the effect of the initial C/N ratio on *Mortierella isabelline* and *Cunninghamella echimulata*. The authors reported an increase in lipid accumulation from 36% to 47% in *Cunninghamella echimulata* and 50% to 56% in *Mortierella isabelline* when the C/N ratio was changed from 83.5 to 133.5. The authors stated that lipid productivity could vary depending on the initial C/N ratio of media and type of oleaginous strain. Similarly, in the present study, the change of C/N ratio from ~300 to 83.5 resulted in high biomass as well as lipid accumulation in *Rhodosporidium toruloides*-1588. However, the lipid production is still low, i.e., 6–7 g/L hence further exploration is needed to improve the lipid production. In addition, with the addition of nitrogen sources, the maximum lipid accumulation time did not change which is quite important for a biorefinery industry. As in the biological process, fast microbial growth, as well as accumulation of products at the early phase experiment, could help decrease the total production time which ultimately helps in reducing the energy consumption and production cost. Similarly, Osorio-Gonzalez et al. [7] reported the maximum lipid accumulation time of different strains of *R. toruloides* was 112–120 h when cultivated in C5 and C6 hydrolysates. On the other hand, Zhao et al. [18] reported 120 h for maximum lipid accumulation when *R. toruloides*-AS 2.1389 was cultivated in detoxified sugarcane bagasse hydrolysate. The present study demonstrated lower than 100 has a higher lipid accumulation time. Figure 3 shows the accumulation of lipids in *R. toruloides*-1588 in hardwood and softwood hydrolysate.
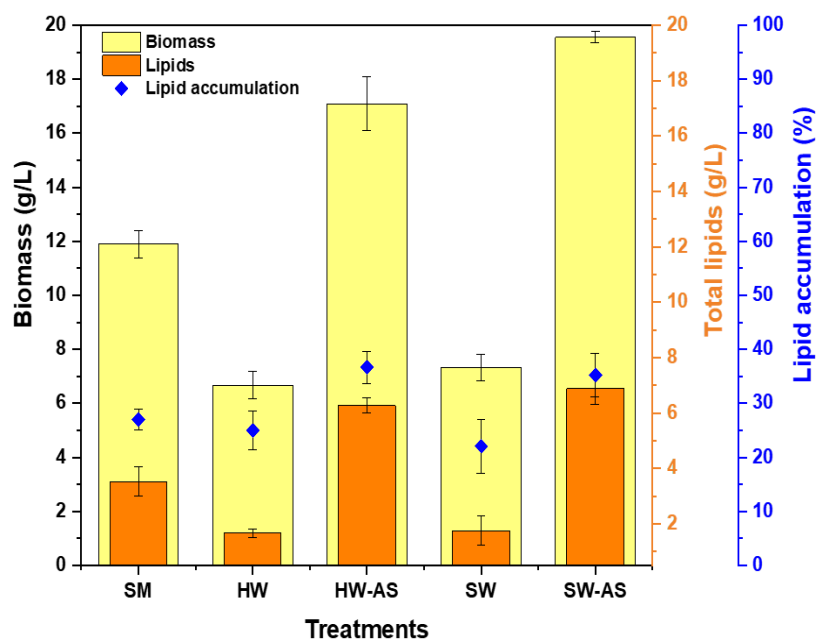

Figure 3. Lipid accumulation profile in synthetic media (SM); hardwood hydrolysate (HW), hardwood hydrolysate + ammonium sulfate (HW-AS); softwood hydrolysate (SW); and softwood hydrolysate + ammonium sulfate (SW-AS).

The microbial lipid composition is generally comprised of carbon atom varying from 10 to 24. The fatty acid distribution can vary depending on the type of strain, culture media, and growth conditions [2]. The fatty acid distribution of lipid was comprised of palmitic, linoleate, oleic, linoleic, and stearic acids, in order of their abundance. Figure 4 represents the fatty acid distribution profile in hardwood, and softwood hydrolysate, as well as in synthetic media. Although the fatty acid composition pattern was the same in the three-culture media (oleic, palmitic, and stearic acid), we observed a peak abundance decrease in synthetic media compared with the obtained abundance in both wood hydrolysates. Wood hydrolysate is a complex medium with a wide amount and presence of compounds that can contribute to the variation in fatty acid composition by increasing stress on the yeast. Similarly, Osorio-Gonzalez et al. [7] stated that concentration and fatty acid composition are

highly dependent on substrate source and media composition. Likewise, Zhao et al. [18] reported a maximum peak abundance of 49%, 6%, and 35% of oleic, stearic, and palmitic acid in a batch experiment using detoxified sugarcane bagasse-derived hydrolysate, while Fei et al. [11] reported 45.2% of oleic acid, 10% of stearic acid, and 25% of palmitic acid in fed-batch conditions using corn stover hydrolysate. The fatty acid compositions of microbial lipid have similarity with plant oils in terms of carbon chains while percentage distribution can vary within the different genera of plants, therefore, it can replace the vegetable oils for biofuel production [2]. For instance, the general fatty acids composition of cocoa butter is 37% oleic acid, 23% palmitic acid, and 32% stearic acid. Therefore, it could be an alternative to cocoa butter [23]. Thus, it can be speculated that (a) lipids formed in *R. toruloides*-1588 in undetoxified hydrolysate could be a promising feedstock in various industrial applications; (b) fatty acid distribution can vary depending on media composition and growth conditions; (c) lipid composition is also dependent on genetic makeup and genera of microorganism.
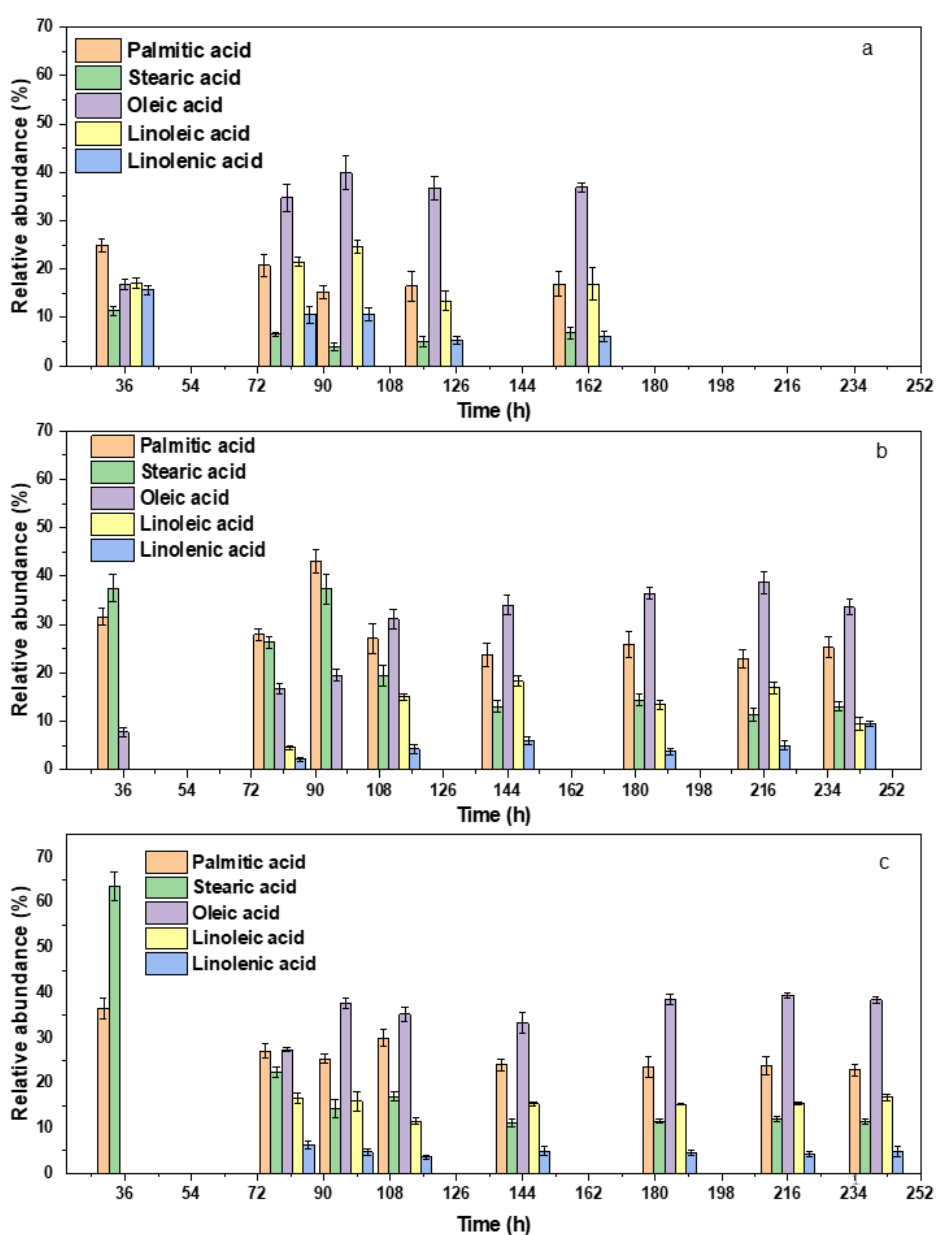

Figure 4. Fatty acid composition of lipid from different growth conditions: (**a**) synthetic media; (**b**) hardwood hydrolysate; (**c**) softwood hydrolysate.

The cetane number of fatty acids was calculated. According to the equation provided by Fei et al. [11], the cetane number (CN) was found to be 49.8, 56.32, 57.12 in synthetic media, hardwood, and softwood hydrolysate, respectively. This result is in accordance with the result reported by Fei et al. [11] with a value of 57.7 of the fatty acid produced using lignocellulosic biomass in *R. toruloides* DSMZ 4444 (Deutsche Sammlung von Mikroorganismen und Zellkulturen). The acceptable value as per the European (EN 14214) and American Society for Testing and Materials (ASTM D6751) is 50 [11].

Consequently, regardless of the low concentration, polyunsaturated fatty acids are quite important, such as docosapentaenoic acid or linoleate due to their high commercial value in chemical, food, and pharmaceutical industries [2]. Table 2 shows the polyunsaturated fatty acid was produced in *R. toruloides*-1588. In general, a low C/N ratio lead to an increase in unsaturated fatty acid production [15]. On the other hand, microbial cells also tend to produce high amounts of unsaturated fatty acids in the presence of inhibitors [7] which could be the possible reason for high polyunsaturated fatty acid production in hydrolysate than in synthetic media grown yeast. This suggested that inhibitors can promote unsaturated fatty acids production. In other words, undetoxified and detoxified hydrolysates also affect lipid compositions, for instance, the unsaturated fatty acid proportion has been observed as slightly lower in detoxified hydrolysate [7]. Conversely, inhibitors can cause total or partial growth inhibition of microorganisms. Likewise, these can interfere with carbon consumption, thus ultimately influencing lipid accumulation.

Table 2. The relative percentage of polyunsaturated fatty acids detected in lipids under different media conditions.

Polyunsaturated Fatty Acids (%)	SM	HW	HW-AS	SW	SW-AS
Alpha linolenic acid	5.21	9.48	2.73	6.29	3.74
Stearidonic acid	1.26	ND	0.68	0.22	0.43
Eicosatrienoic acid	0.71	3.86	ND	2.82	3.76
Docosapentaenoic acid	5.31	5.9	ND	1.98	1.12
Linoleic acid	0.67	4.51	10.05	16.65	9.86
Trans-linoleic acid	ND	0.49	1.12	0.06	0.62
Gamma linoleic acid	ND	0.22	0.29	0.07	0.15
Eicosadienoic acid	0.18	1.06	0.17	0.46	0.36
Dihomogamma linolenic	ND	0.10	0.06	0.09	0.11
Elaidic acid	ND	0.88	1.66	1.09	0.71
Erucic acid	0.72	ND	0.03	0.54	0.24
Eicosenoate	ND	0.93	ND	0.83	ND

SM: synthetic media; HW and HW-AS: hardwood hydrolysate without and with ammonium sulfate; SW and SW-AS: softwood hydrolysate without and with ammonium sulfate; ND: Not detected.

3.4. Effects of Inhibitors

Tolerance and effect of inhibitory compounds on *R. toruloides*-1588 were evaluated throughout the fermentation process. According to obtained results in both hydrolysates, most of the reduction in inhibitors was seen from 80 to 100 h of the fermentation process as shown in Figure 5. The total reduction of syringaldehyde, vanillin, 5-hydroxymethyl furfural (5-HMF), and furfural occurred until 80 h. Furthermore, 32% of the vanillic acid reduction was observed by 72 h. Approximately 20 to 26% of the inhibitor concentration was reduced in control treatment until 200 h of fermentation as shown in Figure 5. The degradation in controls could be due to different fermentation conditions like salt, pH, fermentation time, aeration, or temperature [7]. It has not been established yet that *R. toruloides* can degrade or metabolize inhibitors, but they are known as inhibitor-tolerant species because of overexpression of certain types of genes such as aldehyde dehydrogenase [24]. For instance, NADPH production was increased due to enhanced expression of ribulose 5-phosphate epimerase (RPE1) and phosphogluconate dehydrogenase (GND1) enzymes in the pentose phosphate pathway in *R. toruloides* sp. in the presence of inhibitors [24]. The knockout of GND1 in *Saccharomyces cerevisiae* leads to increased sensitivity towards 5-HMF and furfural [25]. Likewise, aldehyde dehydrogenase 4

(ALD4) is another enzyme that was reported to increase about 3-fold in *R. toruloides*, which involves the conversion of furfural and 5-HMF into furan-2,5-methanol and 2-furan methanol, respectively [26]. Qi et al. [27] reported that if gene expression increases two-fold, it would be considered significant for *R. toruloides* strains. The author demonstrated the change in expression of 39 different genes generally involves in DNA repair, mitogen-activated protein kinase (MAPK) signaling and glycolysis pathway, in the plasma-mutated strain grown on lignocellulosic hydrolysate [27]. The microorganisms capable of degrading furfural and 5-HMF were found to have hmf clusters [28]. Various other genes which were also found responsible for furaldehyde degradation present in the vicinity of hmf are mfc, adh, hyd, and hmf genes. Likewise, *R. toruloides* has also been found to have aldehyde dehydrogenase (adh gene) involved in central metabolism and lipid accumulation but its involvement in furfural degradation is yet to be discovered [29]. These genes could be used as a marker for the tolerance to inhibitor and screening of the tolerant oleaginous strains. Redox potential has also been found to play a crucial role in enhancing furfural tolerance in yeast. Recently, Li et al. [30] studied the furfural degradation using Saccharomyces cerevisiae, when grown in synthetic media (i.e., yeast extract (3 g/L), glucose (100 g/L), peptone (4 g/L), and furfural (4 g/L)) by controlling the extracellular redox potential (ORP), i.e., by adjusting the fermenter aeration. The author reported the maximum biomass growth and furfural degradation was observed at −100 mV ORP while no aeration condition resulted in a prolonged lag phase, lowest biomass, and prolonged furfural degradation time [30]. Hence, it can be suggested that the furfural degradation can be due to oxidation-reduction reaction. Although ORP was not maintained or recorded in the present study, aeration was applied to the culture, hence, this could be another reason for inhibitor degradation by *R. toruloides*-1588.

Inhibitors generally interfere with the normal working of the cellular systems such as the reduction in transport of substrate through the plasma membrane, oxidative stress, variation in the electrochemical gradient of mitochondria, and many more which lead to either reduced growth or growth inhibition, reduced sugar consumption, and variation in bioproduct formation. Hence, it is important to develop or screen the microorganism that could not only deal with high inhibitor but can also produce a high concentration of the desired product. The effect of an inhibitor can also be seen on the FAMEs in the present study. The presence of docosapentaenoic acid and linoleate was detected in a sample collected from hydrolysate in the early exponential phase. Furfural is also reported to affect fatty acid distribution, especially on unsaturated fatty acids [18]. Unsaturated fatty acids are not only required for biofuel but also have high commercial value in nutraceuticals and chemical industries. Adaption of strain in this study and the ability to produce lipids under the presence of inhibitor and without any supplementation in hydrolysate is a promising renewable feedstock to produce microbial lipids. With the addition of ammonium sulfate, a C/N ratio of 87.6 was observed and lipid accumulation increased about 1.5-fold under the presence of a similar amount of inhibitory compounds.

So far, many inhibitory mechanisms of the toxic compounds have been studied and are well-explored [5,17]. Over the past decade, several microorganisms have been identified as capable of degrading the inhibitors. However, these microorganisms would also metabolize monomeric sugars, hence compromising further fermentation procedures. Hence, using a microorganism capable of biological detoxification of wood hydrolysate in addition to the lipid accumulation could be an effective method. Nevertheless, it is advisable to perform further studies aiming at the optimization of the C/N ratio, detailed study of inhibitors degradation, random or directed mutations, to improve the fermentation process that could ultimately lead to high cell mass and lipid titer. Likewise, this study can be a baseline to develop novel detoxification process or pretreatment strategies to obtain the maximum fermentable sugars as a feedstock for microbial lipid production.
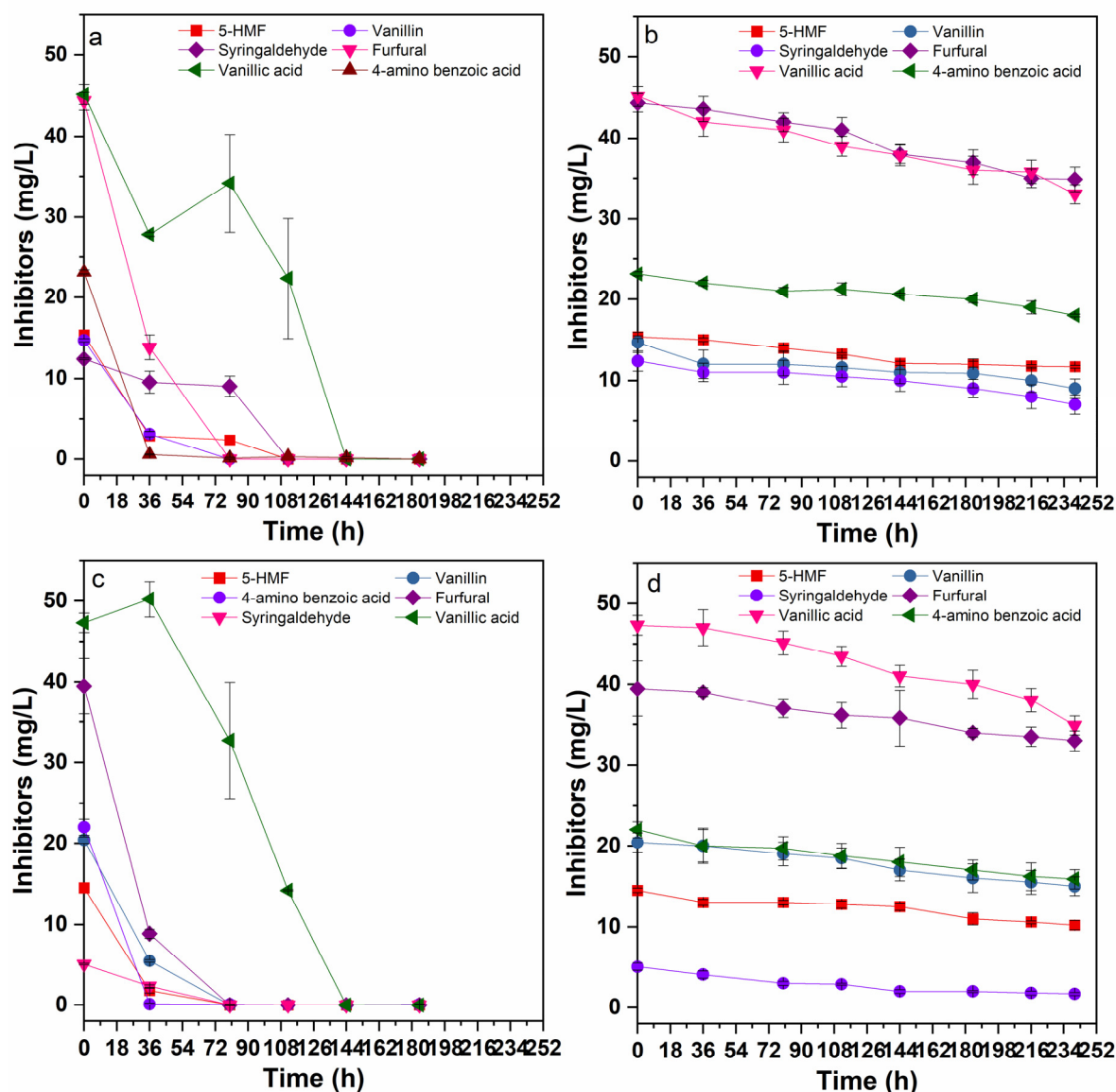
Figure 5. Inhibitor reduction profile in hardwood (HW) and softwood (SW) hydrolysate along with their control: (**a**) HW hydrolysate; (**b**) HW control; (**c**) SW hydrolysate; (**d**) SW control.

4. Conclusions

Forestry biomass as a raw material to obtain liquid hydrolysates for the production of biofuel feedstock was investigated in the present study. *Rhodosporidium toruloides*-1588 exhibited a high amount of cell biomass (17.09 g/L and 19.55 g/L) and a lipid content of 36.68 and 35.24% when it was grown in hardwood and softwood hydrolysate, respectively. A high abundance of oleic (38.69%), stearic (37.4%), and palmitic acid (43.06%) in lipids makes it viable feedstock for advanced biofuel production. Additionally, the evaluated strain showed tolerance to different inhibitory compounds such as furfural (67 mg/L), 5-HMF (25 mg/L), vanillic acid (34 mg/L), and vanillin (7 mg/L), which can further be explored by subjecting the yeast to high inhibitor concentrations and studying their effect on lipid accumulation and fatty acids composition. This study highlights the significance of *R. toruloides*-1588 as a lipid producer using undetoxified and non-supplemented forestry-based wood hydrolysates while setting up the baseline to further explore the stress resistance profile of oleaginous yeast, advanced biofuel production (such as drop-in biofuels) and its improvement using evolutionary engineering.

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