

**Bioprocessing of Fermentable Sugars Derived from Water Hyacinth into Microbial Lipids and Single Cell
Proteins by Oleaginous Yeast *Rhodospiridium toruloides* NCIM 3547**

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Abstract

In this study we employed microwave-acid pretreatment for water hyacinth (WH) to obtain liquid hydrolysate that contains sugars derived from holocellulosic components of biomass for further oleaginous yeast fermentation. In order to remove the inhibitors such as furans after acid treatment, detoxification of hydrolysate was done and we compared the efficiency of this step with non-detoxified hydrolysate towards capability of the *Rhodospiridium toruloides* NCIM 3547 (an oleaginous yeast) to produce microbial lipid and single cell protein. The results indicated that the reducing sugar concentration was found to be higher in non-detoxified hydrolysate (65.41 g/L) than detoxified one (59.18 g/L). When the non-detoxified liquid hydrolysate was supplemented with yeast extract as a complex organic source for *R. toruloides*, resulted in a maximum lipid yield of about 0.813 ± 0.041 (g/g) and 53.60 ± 2.68 (g/g) of single cell protein content with 0.038 g/L/d of protein productivity. Two kinetic models, hybrid Logistic-Monod and Luedeking-Piret were employed to assess the microbial growth and the substrate utilization and were found to be in well agreement with the experimental data with a coefficient of determination (R^2) value ranging from 0.95 – 0.99 thereby demonstrating the efficiency of the hydrolysate supplemented media. Further, GC-MS analysis of transesterified lipids revealed the presence of various FAME (fatty acid methyl esters) and also the presence of increased levels of total saturated fatty acids (35.03%) advocates its high potential in biodiesel production. This study demonstrates the feasibility of sustainable valorization of WH derived liquid hydrolysate towards a greener biorefinery framework.

Keywords: Water hyacinth, Liquid hydrolysate, Waste valorization, Oleaginous yeast, Microbial lipids, Single cell protein

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Conflict of interest

The authors declare no conflict of interest.

Availability of data and material

The figures and the sources of information provided in the manuscript will be provided based on the request.

Code availability

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Author contributions

SJ and VK involved in the conceptualization, planning of the experiment and technical evaluation of the manuscript. SSA, NS, SR, SS and GR have equally contributed in execution of the experiment, data generation, data analysis and drafting of the manuscript. AKC has imparted technical inputs in drafting of the manuscript.

Ethics approval

Not applicable.

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1 Introduction

In recent decades, advancements of greener approaches in the biorefinery framework have gained a tremendous interest as potential alternatives for non-renewable fossil-based chemicals and fuels. The production of fuels and chemicals from renewable feedstock is highly desired due to a number of advantageous features such as sustainability, high energy efficiency, non-toxicity of the process, environmental friendly etc [1,2]. Biodiesel, a mixture of fatty acids alkyl esters, is an important biofuel and its market is growing rapidly as the world is moving towards sustainable and renewable low carbon manufacturing technologies [3]. It has been reported that around 70% of the total production cost of biodiesel are mainly contributed by the cost of the edible vegetable oil (castor, soybean, sunflower and palm) [4]. Currently, the lipids derived from edible biomass termed as first generation feedstock such as vegetable oils and animal fats are used in commercial biodiesel production. However, it is associated with the various shortcomings like food versus fuel debate due to exponentially growing human population, less feasibility for globalization and agricultural land usage [5]. On the other hand, oleaginous organisms (yeast and fungi) can accumulate lipids in significant amounts that reach up to levels exceeding 70% of their total cell biomass. Moreover, the composition of manufactured microbial lipids is comparable to lipids present in vegetable oils used for industrial production of biodiesel [6]. Among the oleaginous organisms, yeast strains are found to be a potent microbial oil producer owing to their unicellular form, short doubling time [7] and ability in utilizing a wide variety of sources such as crude glycerol, industrial wastewater, lignocellulosic hydrolysate and municipal organic wastes [4, 8-11]. *Rhodosporidium toruloides*, is one such promising red oleaginous yeast, considered as GRAS (Generally Regarded As Safe) and natively involved in efficient lipid biosynthesis in terms of lipid amount, yield and productivity [11-13]. It is noteworthy that *Rhodosporidium* species is a robust lipid cell factory as it has a high tolerance for inhibitory compounds present in biomass hydrolysates [12]. Likewise, Tchakouteu et al. [13] investigated the dynamic adaptation and tolerance of *Rhodosporidium toruloides* against sodium chloride enriched high glucose medium for microbial oil production (upto 71.3% w/w) under non-sterile condition. The biochemical behaviour of *R. toruloides* on valorization of waste glycerol towards the synthesis of metabolic compounds like microbial lipids under nitrogen limited condition [14]. As the yeast oil is an intracellular product obtained by the cell wall lysis, another important commercial product that can be expected out of the process is single cell protein from the lysed cell biomass. The life cycle of an oleaginous organism is characterized by three distinct phases namely balanced growth phase, oleaginous and reserve lipid turn over phase. During the initial balanced growth phase, the microbes are primarily involved in metabolizing the excess carbon source

into a cell biomass rich in polysaccharides and proteins with restricted synthesis of lipids that are responsible for its cell membrane construction. Whereas in the oleaginous phase the depletion of nitrogen in the growth medium triggers the de novo lipid accumulation. During this nitrogen starvation, the microbes are continuously involved in assimilating the carbon source for triacylglycerols production. After the carbon source exhaustion, the storage lipids are utilized by the oleaginous cell as an intracellular carbon source for new lipid- free material production or as a maintenance energy source [15]. In most of the yeast strain, nitrogen is responsible for the cell division, nucleic acid and protein synthesis. Thus, in most of the oleaginous yeast strain single cell protein synthesis occurs when the nitrogen source is available however, the lipid accumulation is dominated by the excess carbon source [16].

Water hyacinth (WH) is an aquatic weed containing high amount of cellulose (24.7%) and hemicellulose (32.2%) with less lignin content (3.2%) [17-20]. Widespread of this biomass on water surface has created numerous ecological problems like destruction of aquatic flora and fauna, irrigation problems as well as caused several vector borne diseases in humans [21,22]. Thus, the demand for eradication and valorization of WH has grown rapidly in recent years and resulted in its various applications like biomanure [22], bioethanol [18, 23] and biogas production [24,25]. However, utilization of WH for yeast based lipid production has not been reported so far. The current study was undertaken to examine the ability of yeast *R. toruloides* NCIM 3547 to accumulate lipids and proteins on WH hydrolysate rich in fermentable sugars. The work started with characterisation of WH followed by its pretreatment using microwave-acid method. The liquid hydrolysate rich in sugars was detoxified and performance of the yeast was compared before and after the detoxification. The lipids and proteins accumulated by the yeast grown on non-detoxified and detoxified hydrolysate were quantified. The lipids were also characterised using GC-MS. To the authors' best knowledge, this is the first report on lipid and single cell protein production from WH.

2 Materials and methods

2.1 Lignocellulosic feedstock and microorganism

WH was collected from the nearby local lake of SRM Institute of Science and Technology, Tamil Nadu, India. After harvesting, the plants were washed thoroughly with the running tap water to remove the debris. Then, the biomass was subjected to solar drying (48 h) until considerable moisture removed, thereafter oven drying (60 °C) until constant weight is attained and pulverized to obtain a coarse powder of 0.2 mm particle size and subsequently stored at room temperature in zip lock cover for further treatment.

R. toruloides NCIM 3547, an oleaginous yeast [26,27] was procured from National Collection of Industrial

Microorganisms (NCIM) designated as a microbial resource center of CSIR- National Chemical Laboratory, Pune, Maharashtra, India. The microbial strain was grown and periodically sub-cultured on a yeast extract peptone dextrose (YEPD) media (yeast extract- 10 (g/L); peptone- 10 (g/L); dextrose- 20 (g/L) and agar- 20 (g/L)) followed by maintaining the culture at 4°C for later use.

2.2 Characterization of WH

According to the standard American Public Health Association (APHA) protocol, the proximate analysis (moisture content, volatile solids, total solids, and ash content) of WH was performed [28]. The structural composition such as cellulose, hemicellulose and lignin content of WH were determined using protocol as reported by Sun et al. [29]; Marlett and Lee [30] and Hussain et al. [31] respectively. Further, elemental composition of WH such as carbon, nitrogen, sulphur and hydrogen were performed using CHNS/O Elemental analyzer, Perkin- Elmer 2400 series, US.

2.3 Microwave-acid hydrolysis of WH

Microwave-acid hydrolysis of WH was carried out using laboratory microwave oven (LG, model MC2149BPB, India) that operates up to a maximal microwave radiation of about 0 W. Dilute sulphuric acid (2.5%, v/v) was added to 10% (w/v) of WH and subjected to microwave irradiation at 160 W for 7 minutes [32]. Then, the liquid hydrolysate was separated by centrifugation at 5000 rpm for 10 min followed by filtration and finally stored at 4°C for later use. The liquid hydrolysate obtained after microwave-acid treatment of WH was divided into two fractions and subjected to the preparation of detoxified and non-detoxified liquid hydrolysate for removal of inhibitory production as given in section 2.4.

2.4 Detoxification and non-detoxification of WH hydrolysate

Detoxified water hyacinth hydrolysate (DWHH) was obtained by a process known as over liming. First, the liquid hydrolysate was heated at 42 °C in a magnetic stirrer hotplate and the potassium hydroxide (5 N) was added slowly until it reached pH-10 and temperature $50 \pm 2^\circ\text{C}$. Later, the DWHH was maintained at 50 °C with constant stirring for about 30 min followed by filtration using a muslin cloth. Subsequently, the cooled DWHH was re-acidified with sulphuric acid (pH-5.5) and the precipitates were removed by the filtration. Whereas, in case of non-detoxified water hyacinth hydrolysate (NDWHH), 5 N potassium hydroxide solution was added to a fraction of liquid hydrolysate obtained after microwave-acid treatment until the pH reaches 5.5, followed by filtration using muslin cloth [33]. The obtained DWHH and NDWHH were further used as a substrate for lipid fermentation using *R. toruloides* NCIM 3547.

2.5 Inoculum preparation and single cell oil production

R. toruloides NCIM 3547 with an optimum pH range- 4 to 7.5 [34-37] was grown in a 100 mL Erlenmeyer flask containing 50 mL of YEPD broth and the culture was maintained at 30°C, pH-7.0 in an orbital shaker operated at an agitation speed of 250 rpm. After 24 hours of incubation, 10% (v/v) of the fresh seed inoculum with optimal density of about 0.1 (OD_{600nm}) was added to the fermentation medium containing 25 mL of either DWHH or NDWHH along with the minimal media components (g/L): CuSO₄.5H₂O-0.0001, KH₂PO₄-2, MnSO₄.H₂O-0.003, MgSO₄.7H₂O- 0.4, and yeast extract-1.5. The fermentation sets such as DWH H, NDWHH and the control sets (DWHH (C) and NDWHH (C) i.e., without the addition of supplement (yeast extract)) were autoclaved. The fermentation was carried out in a 100 mL Erlenmeyer flask incubated at 30°C under constant shaking (250 rpm) for a period of 7 days [33].

2.6 Lipid and protein estimation

Microbial lipids from all the fermentation sets (DWHH, NDWHH, DWHH (C) and NDWHH (C)) were extracted according to Bligh and Dyer protocol [38] with some modification. About 20 mL of culture (after 168 h) was centrifuged at 6000 rpm for 10 mins at 4°C; the cell pellet obtained was washed twice with distilled water and allowed to dry until a constant weight is reached. To the dried cell biomass, a mixture of 10 mL chloroform:methanol (2:1) and glass beads (5-10) were added and vortexed for 30 min. Followed by sonication in a water bath sonicator (70 Hz and 40 min) and centrifugation at 6000 rpm at 4°C for 10 min. Finally, the supernatant was mixed with 10 mL of chloroform:methanol mixture and vortexed for 5 min, where the impurities has been removed by the addition of 3 mL of 0.9% (w/v) NaCl. The samples were then subjected to phase separation by incubating it for about 10 min, where the organic phase containing lipid was recovered and subjected to evaporation to remove the solvents. The total lipid content was weighed and expressed as a percentage of the extracted lipid on the dry cell biomass (g lipids/g biomass). Lipid concentration of DWHH, NDWHH, DWHH (C) and NDWHH (C) was calculated using Eq. (1).

$$\text{Lipid concentration } \left(\frac{g}{L} \right) = \frac{\text{Lipid dry weight (g)}}{\text{Volume of sample (L)}} \quad 1$$

In addition to this, the cell pellets obtained during lipid extraction were also used to estimate the protein content using Lowry method [39].

2.7 Cell mass and reducing sugar estimation

During the microbial lipid production, 3 mL of the cell suspension was periodically collected for every 24 hours and the microbial growth was determined by measuring the optimal density at 600 nm followed by centrifugation at 6000 rpm at 4°C for 10 min. The cell pellet was dried in pre-weighed centrifuge tube at 80°C until constant weight is attained

and the final cell biomass concentration was expressed in terms of g dry cell weight (DCW)/L. Simultaneously, the supernatant obtained after centrifugation was subjected to the reducing sugar analysis by 3,5-dinitrosalicylic acid (DNS) method [40].

2.8 Kinetic modeling

An attempt has been made to analyze the influence of different carbon sources on cell growth (biomass) and the substrate utilization of *R. toruloides* NCIM 3547 using hybrid Logistic-Monod and Luedeking-Piret equations respectively [41]. A non-linear regression method was used to solve the kinetic model equation and was fitted into the experimental data to estimate the model kinetic parameters. The data obtained were analyzed using MS Office Excel 2013 software (Solver toolkit) through the Levenberg-Marquardt algorithm in order to minimize the residual sum of squares of errors. As described by Xu [42], it has been suggested that when the Monod Model (based on limiting substrate) is combined with Logistic Model (based on growth inhibitors or toxic chemicals accumulated other than limiting nutrients) there is a possibility in understanding the cell physiology. In this study, the hydrolysates utilized as the growth medium for *R. toruloides*, it is obvious that there might be presence of both the monosaccharides for the growth of microbes along with inhibitors (resultant from the acid treatment) might influence the growth. Therefore, this hybrid model equation was employed (Eq. 2) as an unstructured kinetic model to analyze the *R. toruloides* fermentation process effectively. The kinetic parameter associated with the *R. toruloides* NCIM 3547 growth (X_0 , X_{max} , and μ_{max}) and the substrate utilization (S_0 , α , and β) were determined using Eq. (2) and (3) respectively as given below,

$$X = \frac{X_0 \times X_{max} e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}} \quad (2)$$

Where, X (g/L) – cell biomass concentration at any time t (h); μ_{max} (h^{-1}) - maximum specific growth rate; X_{max} (g/L) - maximum cell biomass concentration in the fermentation medium; X_t and X_0 - cell biomass during fermentation $t=t$ ‘h’ and 0 ‘h’ respectively

$$S = S_0 - \left(\frac{n}{Y_{P/S}} + K_e \right) \frac{X_{max}}{\mu_{max}} \left[\left(\mu_{max} t + \ln \left(\frac{X_0}{X_t} \right) \right) \right] - \left(\frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}} \right) (X_t - X_0) \quad (3)$$

Based on the kinetic parameter obtained through the modelling, the mode of lipid accumulation in the given microbe with supplied DWHH and NDWHH was analysed from Eq. (4) and (5).

$$\alpha = \frac{1}{Y_{X/S}} + \frac{m}{Y_{P/S}} \quad (4)$$

$$\beta = \frac{n}{Y_{P/S}} + K_e \quad (5)$$

Where, S_0 - initial substrate concentration at time $t = 0$; $Y_{X/S}$ (g cell biomass produced/g sugar consumed) - yield coefficient for cell biomass; $Y_{P/S}$ (g lipid produced/g sugar consumed) - yield coefficient for lipid; K_e - maintenance coefficient; α -growth associated factor; β - Non growth associated factor; If $\alpha > \beta$ - growth associated product formation, $\alpha < \beta$ - non-growth associated product formation.

2.9 GC-MS and HPLC analysis

The dried crude lipid residues obtained from different carbon sources were treated with 2:1 ratio of n-hexane and methanolic KOH (2 M) and incubated at 70°C for about 20 min. After cooling, 1.2 mL of 1 M HCl was added with gentle stirring. Followed by addition of n-hexane (1 mL) for the phase separation. The upper aqueous layer containing the FAME has been subjected to gas chromatography mass spectrometry (GCMS- Shimadzu QP2010 system, Japan) equipped with Sh-Rtx-5 column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m). The column temperature was maintained at 50°C to 300 °C at a rate of 7.5°C/min and hold for 2 min. Helium as carrier gas was provided and maintained at a constant flow rate of about 3.0 mL/min with 1:10 split ratio and the data obtained were evaluated using NIST/EPA/NIH Mass Spectral Library (NIST 14) and NIST Mass Spectral Search Program (Version 2.2).

HPLC analysis of WH hydrolysate was performed to detect the inhibitors and sugars before and after detoxification. The HPLC system 1260 (Agilent Technology, Infinity series, US) with C18 column (4.6 $\times$ 250 mm $\times$ 5 μ m) was used for analysis furfural (column temperature-40°C, flowrate-1.0 mL/min, mobile phase -water:methanol (1:1, 50%, v/v), 5-hydroxymethylfurfural (column temperature-35°C, flowrate-0.8 mL/min, mobile phase-water: methanol (1:1, 20%, v/v)) and syringaldehyde (column temperature-25°C, flowrate-0.7 mL/min, mobile phase-formic acid:acetonitrile (1:1, 0.1%, v/v)). An injection volume of 20 μ L for standards and hydrolysate sample was maintained using methanol as a solvent.

3 Results and discussion

3.1 Biochemical characterization of water hyacinth

Generally, the relative abundance of major structural components of various lignocellulosic biomass i.e., cellulose, hemicellulose and lignin predominantly depend on the origin of the plant material [43]. The proximate and biochemical composition analysis of WH revealed (Table 1) that cellulose fraction (31.55%, w/w) is the major component of WH

followed by hemicellulose (24.13%, w/w) with a little amount of lignin (5.15%, w/w). Thus, the presence of high content of holocellulosic components (55.68%, w/w) (i.e., cellulose and hemicellulose) paved the way for the exploiting the WH hydrolysate (rich in fermentable sugars) in microbial lipid and single cell oil production. Lignin is a cumbersome component and high lignin content impedes the saccharification process. Therefore, low lignin feedstock is highly desired for microbial bioconversions as it enables the efficient hydrolysis which in turn enhances the recovery of reducing sugar yields. Additionally, during hydrolysis the low amount of lignin minimizes the formation of phenolic derivatives and its inhibition in microbial proliferation [33,44]. Further, the presence of high moisture content (92.6%, w/w) and volatile solid content (97.3%, w/w) of WH indicates that the structural components cellulose and hemicellulose are highly available as raw materials for biorefinery and bio-based conversion routes. As reported, based on the season of harvest, geographical location and the plant cell wall maturity, the relative composition of the structural and elemental characteristics of WH in this present study varies slightly with the previous studies [24,25,45,46].

3.2 Effect of microwave-acid pretreatment on reducing sugar

An efficient hybrid pretreatment strategy i.e., microwave-acid has been adopted for WH in order to facilitate the solubilization of lignin, reduce formation of toxic fermentation inhibitors and improve the reducing sugar yield [47]. After the microwave-acid pretreatment of WH, the liquid hydrolysate was subjected to overliming (detoxification process) to remove the undesired phenolic and furan derivatives. The total reducing sugar concentration of detoxified (DWHH) and non-detoxified (NDWHH) hydrolysates of WH were found to be 59.2 g/L and 65.4 g/L respectively. The reducing sugar obtained after hybrid pretreatment strategy (microwave-acid pretreatment) of WH was found to be higher than the chemical pretreatment (dilute acid) of various biomass rice straw (35.2 g/L) and corn cob (45.7 g/L) [32,48]. Thus, the microwave-acid pretreatment enhances the reducing sugar concentration as compared to the chemical pretreatment. However, hydrolysis of cellulose and hemicellulose component could be enhanced by enzymatic hydrolysis of pretreated substrate using cellulolytic/hemicellulolytic enzymes respectively that allow the complete utilization of available carbon source [49]. Thus, the enzymatic saccharification of pretreated substrate facilitates the release of fermentable sugars thereby, improving the C:N ratio for enhanced lipid yield [50]. Similar to the previous studies, the reducing sugar concentration of detoxified hydrolysate (DWHH) was lower than the non-detoxified hydrolysate [10,51,52] which might be due to the reducing sugar destruction at the elevated pH during the detoxification [53,54]. The loss in amount of reducing sugar after detoxification of WH hydrolysate was found to be

9.52% which was less comparable to the sugar loss observed in the acid pretreated sugarcane bagasse hydrolysate (13.89% to 14.70%) [52].

3.3 Effect of microwave-acid pretreatment on cell biomass production

Detoxified and non-detoxified liquid hydrolysate of WH has been used as a carbon source for the growth of *R. toruloides* NCIM 3547 and concomitant lipid accumulation. This is done to verify the extent of the microbial innate potential to withstand the inhibitory products. Here, one fraction of detoxified and non-detoxified liquid hydrolysate was supplemented with yeast extract while another fraction is devoid of the supplement and the purpose of exclusion of yeast extract is to examine the role of the DWHH and NDWHH as sole carbon source for the *R. toruloides* growth. It is based on the hypothesis that liquid hydrolysate being supplemented with the yeast extract as a complex organic source was able to provide the required nutrients for the *R. toruloides* NCIM 3547 to sustain the growth. The growth pattern of *R. toruloides* NCIM 3547 on four different carbon sources namely DWHH, NDWHH, DWHH (C) and NDWHH (C) was observed over a period of 7 days as shown in Fig. 1 (a) and (b). It was observed that the presence of supplements in DWHH and NDWHH enhanced the microbial growth as compared to the control sets (DWHH (C) and NDWHH (C)). From the Fig. 1 (a) and (b), an initial lag phase was observed within 24 h in all the carbon source, where the cell biomass yield calculated based on DCW was found to increase rapidly during the exponential growth phase 0.79 g/L to 16.14 g/L (NDWHH), 0.73 g/L to 6.92 g/L (NDWHH (C)), 0.52 g/L to 11.47 g/L (DWHH), 0.68 g/L (DWHH (C)) within 120 h and then attained stationary phase (144 h - 168 h). On the other hand, the lag phase of *R. toruloides* NCIM 3547 in DWHH was observed to be longer than the NDWHH which might be due to some physiological stress and thus in turn renders the NDWHH as a more suitable substrate for the yeast metabolism [52,53]. During the exponential growth, the cell biomass (DCW) yield of NDWHH (16.58 g/L) was found to be higher than the NDWHH (C) - 7.03 g/L. However, the detoxified liquid hydrolysate showed an insignificant difference in cell biomass yield between DWHH and DWHH (C). When pure glucose is used as a carbon source, the cell biomass yield of *R. toruloides* was found to be around 37 g/L as reported in previous studies [36]. The highest cell biomass concentration was observed in NDWHH (16.58 g/L), DWHH (11.87 g/L) which was nearly similar to the *C. curvatus* DCW 17.2 g/L and 15.6 g/L when cultured in the non-detoxified and detoxified hydrolysate of wheat straw respectively [33]. The possible reason could be the presence of a higher amount of reducing sugar in NDWHH than DWHH, the inhibitor tolerance of the yeast strain in NDWHH that are attributed by larger inoculum size [56] or the inherent capabilities of the yeast strain [32]. And also, the presence of KOH added during the detoxification of WH hydrolysate

and its subsequent neutralization to pH 5.5 using 1 N sulphuric acid would be a reason for reduced lipid concentration in DWHH.

3.4 Influence of carbon source on lipid production

For the sugar-based substrates, the oleaginous yeasts adopt de novo pathway for lipid synthesis where acetyl and malonyl-CoA are used as a building block for de novo lipid accumulation [57]. In the present study, after 168 h of fermentation a maximum lipid concentration (2.62 g/L), lipid yield (0.81 g/g) and lipid productivity (0.37 g/L/d) were observed in NDWHH as shown in the Fig. 2 (a) and (b). This is in parallel with the cell growth exhibiting the maximum sugar uptake rate (0.019 g/L/h) and sugar utilization efficiency of about 94.55% (59.185 g/L to 3.225 g/L) at the end of fermentation as compared with the other fermentation sets. The total lipid yield is directly correlated with the DCW of the oleaginous yeast, where the overall highest lipid content is achieved for more cells with similar lipid content [58]. The lipid concentration (Fig. 2 (a) and (b)) of all the non-detoxified fermentation sets (NDWHH and NDWHH (C)) was found to be slightly higher than the detoxified ones (DWHH and DWHH (C)). It indicates that the oleaginous strain was able to grow and accumulate lipids thereby the non-detoxified hydrolysates did not show any inhibitory effect on the microbial growth [33]. On the other hand, the lipid content of NDWHH (C) was found to be 32.24 g/g where *R.toruloides* yield around 36% of lipid from the wood hydrolysate owing to presences of other constituents of hydrolysates. Also, the lipid concentration of NDWHH (C), DWHH and DWHH (C) which is similar to the other reported studies [59,60]. The hierarchy of the sugar utilization efficiency was found to be NDWHH (94.55%) > DWHH (82.83%) > NDWHH (C) (82.05%) > DWHH (C) (81.47%) indicates that varying a tolerance of yeast towards inhibitors as exhibited by *Trichosporon fermentans* in rice straw hydrolysate fermentation [32]. Here, on the depletion of bioavailable nitrogen, the reducing sugar from the WH hydrolysate would be regulated into lipid production by *R.toruloides* strain. During this phase, reducing sugar was directly metabolized into acetyl-CoA by inhibiting the TCA cycle via ATP:citrate lyase and thus the acetyl-CoA formed was utilized for oxaloacetate and fatty acid synthesis by oleaginous yeast [6,61]. Based on this study, WH hydrolysate without detoxification could serve as an appropriate substrate for efficient lipid production. Thus, the additional detoxification step could be eliminated which in turn decreases the overall production cost. Further, optimization of lipid fermentation could enhance the lipid production using *R.toruloides*. A comparison table indicating the lipid accumulation using different lignocellulosic sugars including WH along with *R. toruloides* and other oleaginous microbes are represented in Table S3 (supplementary data).

3.5 Kinetic model analysis for microbial growth and substrate consumption

Kinetic model favors in understanding of the non-linear behaviors of the biological reactions and estimates the performance under different operational conditions. Two different models have been employed for their suitability of application to these selected substrates and determine certain kinetic constants that could be helpful for further scale up of the fermentation process. The growth kinetic analysis of *R.toruloides* strain was performed using hybrid Logistic-Monod equation and the results obtained were represented in Table 2. The kinetic model prediction for initial cell growth (X_0) and the maximum cell growth (X_{max}) of *R.toruloides* strain (lag phase to stationary phase) were found to be consistent with the experimental result as deciphered in Fig. 3 (a), (b), (c) and (d). However, the maximum specific growth rate (μ_{max}) was higher in case of DWHH (C) (0.066 h^{-1}), followed by 0.065 h^{-1} , 0.059 h^{-1} , 0.056 h^{-1} for NDWHH (C), DWHH, NDWHH respectively. The μ_{max} value obtained was higher than the value observed during the growth of *R. glutinis* using different carbon substrates (0.05 h^{-1}) [41]. Also, the μ_{max} obtained was in the similar range of those obtained from *R. toruloides* during growth on mandi waste extract [62]. From Table 2, it was observed that the specific growth rate of *R.toruloides* strain in NDWHH was lower than the other carbon sources, yet maximal cell biomass production was found to be 18.31 g/L . This might be due to the high substrate utilization rate by *R. toruloides* in NDWHH and presence of yeast extract as a vital nutrient supplement. The result was evident from the previous study by Gonzalez-Gracia et al. [63] where the maximal cell biomass production (39.28 g/L) was obtained for the fermentation set with lower μ_{max} value (0.025 h^{-1}). On comparison with other substrates having higher μ_{max} value, the cell biomass production and yield is predominantly dependent upon the extrinsic factors like nutrient limitations and fermentation conditions.

On the other hand, the kinetic modeling analysis for the substrate utilization efficiency of *R. toruloides* strain was performed and the results were tabulated in Table 2. From the table, *R. toruloides* growth in NDWHH was able to utilize the maximum substrate (reducing sugar) and also the kinetic parameter like $Y_{x/s}$ (0.45) and $Y_{p/s}$ (0.20) were higher as compared to the other carbon hydrolysates. Thus, the result obtained through model prediction for substrate utilization indicates that substrate consumption by *R. toruloides* strain mainly depends on its growth. Thus, the reducing sugar was consumed by the *R. toruloides* strain in order to sustain its growth and maintenance along with the product formation. The kinetic parameters obtained from the present study were almost equal to the reported values where silkworm pupae hydrolysate was utilized as a substrate for *Yarrowia lipolytica* strain for lipid accumulation [64]. The α and β factors in the kinetic model represent the growth-associated and non-growth associated product

formation constants respectively. Yeast prioritizes the utilization of supplied C source towards (a) growth (lipid free biomass, X); (b) maintenance to requirements. Thereafter, the shift in utilization of C for lipid accumulation happens that makes the yeast as 'Oleaginous', apart from basal quantity of lipid synthesis for cell membranes [65]. To elucidate the role of maintenance coefficient in product synthesis, α and β factors have been included in the Eq. 3. It was observed that $\alpha > \beta$ for all the hydrolysate substrate which shows that the lipid accumulation and lipid synthesis of *R. toruloides* strain would be a growth-associated process. Further, it was confirmed by the extended log phase as represented in Fig. 1 (a) and (b). Similarly, Zeng et al. [66] reported that α value (0.22 g/g) was higher than the β value (0.004 g/g/h), when *R. toruloides* was used for fermenting the food waste hydrolysate. All the kinetic parameters obtained through model prediction for microbial growth and the substrate utilisation were well agreed with the experimental data with a coefficient of determination (R^2) value ranging from 0.95 – 0.99.

3.6 Influence of carbon source on single cell protein production

The comparison of protein productivity, concentration and content of the all carbon sources fermented by *R. toruloides* strain are represented in Fig. 2 (c) and (d). Generally, the protein percentage in any oleaginous yeast strains ranges between 47% to 53% [67]. A maximum protein content of about 53.60 g/g was observed for NDWHH with 0.038 g/L/d of protein productivity as compared with other carbon sources. The result obtained was consistent with study reported by Magalhães et al. [68] where 60.50% of protein was observed in yeast biomass of *Candida tropicalis* on fermenting with sugarcane bagasse hydrolysate. The yeast biomass with protein percentage greater than 40% are considered as a potent single cell protein producer. Zeng et al. [66] reported the capability of *R. toruloides* Y2 for the potential generation of single cell protein where this microbial protein could be applied as a human and animal feed additive. Similarly, the protein percentage of DWHH and DWWH (C) was comparable with the other reported studies 21.7% (food waste hydrolysate), 20-24.5% (fish) [66,69]. When the nitrogen supply is limited, the yeast strain attains stationary phase of growth (120-168 h) and then the excess carbon source in the hydrolysate shifts the metabolic flux towards energy storage mechanism resulting in the single cell lipid and single cell protein accumulation [70,71]. Based on this study, *R. toruloides* NCIM 3547 has the potential ability to metabolize the liquid hydrolysate of WH for the concurrent production of single cell lipids and protein, thereby paving the biorefinery framework for lignocellulosic biomass. This could elucidate the potential of *R. toruloides* to be a potent candidate for single cell oil and protein production from the WH hydrolysates. Further, the single cell protein synthesis could be enhanced by optimizing the fermentation conditions for its commercial applications. Recently, Wang et al. [70] investigated the co-production of

single cell lipid and protein in fed batch fermenter by *Sporidiobolus pararoseus* under ammonia nitrogen limited conditions where, lipid concentration and productivity was found to be 47.1 g/L and 0.66 g/L/h respectively.

Under nitrogen deficient conditions, isocitrate dehydrogenase activity is found to be low owing to its adenosine monophosphate (AMP-allosteric regulator) diminishing thus shifting the metabolic flux towards intracellular lipid synthesis and accumulation. On the other hand, yeast cells also catabolize the AMP to produce ammonium to generate an internal nitrogen source and regulate the amino acid metabolism [72].

3.7 GC-MS analysis for fatty acid composition

The fatty acid profiles from the extracted microbial lipids of different carbon sources namely NDWHH, NDWHH (C), DWHH and DWHH (C) after 7 days of fermentation were represented in Fig. 4. Based on the previous study, all the fatty acids (saturated and unsaturated) could be transesterified into biodiesel-like, where the presence of oleic acid are found to be efficient fatty acid which improves the fuel performances. Fig. S1 (Supplementary data) reveals that the major fatty acid methyl esters from transesterified lipids that include lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid and oleic acid. The hierarchy of saturated and unsaturated fatty acid profiles based on relative composition by area percentage (Table S1 (Supplementary data)) are as follows: lauric acid (C12:0- 16.01%) > stearic acid (C18:0-6.62%) > palmitic acid (C16:0-6.23%) > myristic acid (C14:0-5.15%) > oleic acid (C18:1-4.26%) > elaidic acid (C18:1-3.6%) > pentadecanoic acid (C15:0-1.02%) > erucic acid (C22:1-0.3%) > palmitoleic acid (C16:1-0.06%). From Fig. 4, the presence of increased level (35.03%) of total saturated fatty acids (C18:0, C16:0, C14:0, C12:0, C15:1) in the transesterified lipids could improve the biodiesel quality such as oxidative stability, nitrogen oxide level and octane number. Whereas, the cold flow property of biodiesel is enhanced by the presence of lower level of total unsaturated fatty acids (8.22%) [41,73]. The composition of the fatty acid methyl ester obtained in this study was comparable to the transesterified fatty acid profiles of food waste hydrolysate, cassava starch hydrolysate and rapeseed oil [65,58,74,75]. Further, relative composition of the total saturated fatty acids of DWHH are found to be higher (20.58%) (Fig. 4) and this result was consistent with the maximum amount of lipid content (33.65 g/g) obtained in the DWHH as compared to other samples. Whereas, NDWHH showed a maximum relative composition of about 6.56%, which indicates that *R. toruloides* NCIM 3547 was also able to utilize non-detoxified substrate and efficiently accumulate lipid as similar to detoxified one. Amirsadeghi et al. [41], reported that the composition of fatty acids primarily depends on the growth condition of oleaginous yeast and its medium type.

3.8 HPLC analysis of WH liquid hydrolysate

Lignocellulosic biomass derived compounds such as lignin derivatives (syringaldehyde) and furan derivatives (furfural and 5-hydroxymethylfurfural (HMF)) were generally generated during acid hydrolysis. These are the major compounds that show inhibitory effects on the microbial growth during fermentation process thereby hindering the efficacy of its bioconversion. Thus, in the present study, the liquid hydrolysate obtained after microwave-acid pretreatment of WH was subjected to an over liming process (detoxification) in order to reduce/eliminate the inhibitory effects of the lignocellulosic derived toxic compounds. The detoxified and non-detoxified liquid hydrolysate of WH was subjected to the quantitative HPLC analysis to detect the presence of inhibitors such as furfural, 5-HMF and syringaldehyde. The single point calibration was performed for the standard samples at a concentration of 100 µg/mL. Based on that, the response factor has been calculated from the peak area and standard concentration. The concentration of inhibitors in NDWHH and DWHH samples were calculated by dividing the peak area of respective samples by the response factor. The concentration and the chromatogram of inhibitors before and after detoxification were represented in Table S2 (Supplementary data)) and Fig. S2 (Supplementary data).

The concentration of the lignocellulose degradation by-products is directly related to the pretreatment type, the source of lignocellulosic biomass and its influence on the microbial cell. From Table S2, it was evident that the presence of highly toxic inhibitors such as furfural and HMF in the DWHH and NDWHH are pentose and hexose degradation by-products. These inhibitory compounds affect the specific growth rate of microbial cells and inhibit the precursor molecule production for fatty acid synthesis [76]. In molecular pathway of *R. toruloides*, it inhibits the enzymes (hexokinase, alcohol dehydrogenase, glyceraldehyde 3- phosphate dehydrogenase, pyruvate dehydrogenase and aldehyde dehydrogenase) involved in formation of acetyl CoA from pyruvate. Whereas, 5-HMF causes the amino acid assimilation thereby affecting the cellular respiration mechanism in microbes [77]. Though the concentrations of toxic inhibitors are found to be higher in NDWHH than DWHH, the maximum cell biomass yield and lipid concentration was observed for NDWHH than DWHH. This might be due to the inherent ability of *R. toruloides*, or high amount of fermentable sugars. However, the detoxification has resulted in the 2.22-fold and 1.36-fold decrease in the WH hydrolysate that might reduce the negative effect of inhibitors on production of lipids, where lipid content of DWHH was higher than NDWHH as shown in Fig. 2. And also, the presence of low molecular weight phenolic compounds like syringaldehyde in DWHH and NDWHH affects the selective barrier and enzyme matrices of the *R. toruloides*, thereby inhibiting the fermentation by decreasing the sugar assimilation, cell growth and causing an alternation in the

electrochemical gradient across mitochondria [54]. The concentration of syringaldehyde was decreased by 14.54-fold when the WH hydrolysate was subjected to detoxification. In comparison, the concentration of toxic inhibitors obtained in the liquid hydrolysate of WH during microwave-acid pretreatment was less than the inhibitors present in sugarcane bagasse, wheat straw and corn stover hydrolysate derived after dilute acid hydrolysis [12, 33,78].

From the HPLC chromatogram (Fig. S3 (supplementary data)), was observed that arabinose, mannose and glucose were the major monosaccharides in both NDWHH and DWHH. The concentration of hexose (mannose and glucose) was 4.3 times higher than the pentose (arabinose) in WH hydrolysate before and after detoxification. The overliming of WH hydrolysate has resulted in 32.91% of arabinose, 3.62% of mannose and 51.22% of glucose. A concentration of 28.40 g/L of total fermentable sugar in WH derived hydrolysate was observed after pretreatment, whereas after detoxification a total loss of about 34.19% was detected as the pH of WH hydrolysate was increased to pH 10. The glucose concentration was found to be higher (14.97 g/L), while the arabinose concentration was lower (5.29 g/L). The fermentable sugar obtained from WH hydrolysate after microwave-acid pretreatment was comparable with the acid hydrolysate of sugarcane bagasse (30.29 g/L) and olive tree residues (25.23 g/L) [79,80].

4 Conclusion

This study demonstrated the bioprospection of WH derived hydrolysate rich in fermentable sugars for concurrent production of microbial lipids and single cell protein by *R. toruloides*. However, implications of acid pretreatment through inhibitors is found to be constraint that has been alleviated by adopting detoxification with a compromise in loss of fermentable sugars. Considering the advantage of recoverable sugars for fermentation DWHH could be an effective nutrient supplement instead of cost incurring yeast extract. Elucidation of kinetic behavior of the yeast through adoption of hybrid Logistic-Monod and Luedeking-Piret equations demonstrated the suitability of the hydrolysate as a growth medium equivalent of conventional production media. Microbial lipid derived from *R. toruloides* with more saturated fatty acids (35.03%) suggested as a probable candidate for biodiesel production. Thus, the hydrolysate derived from WH could be a promising feedstock for biorefinery purposes from a circular bioeconomy perspective where an aquatic menace could be converted into energy crop.

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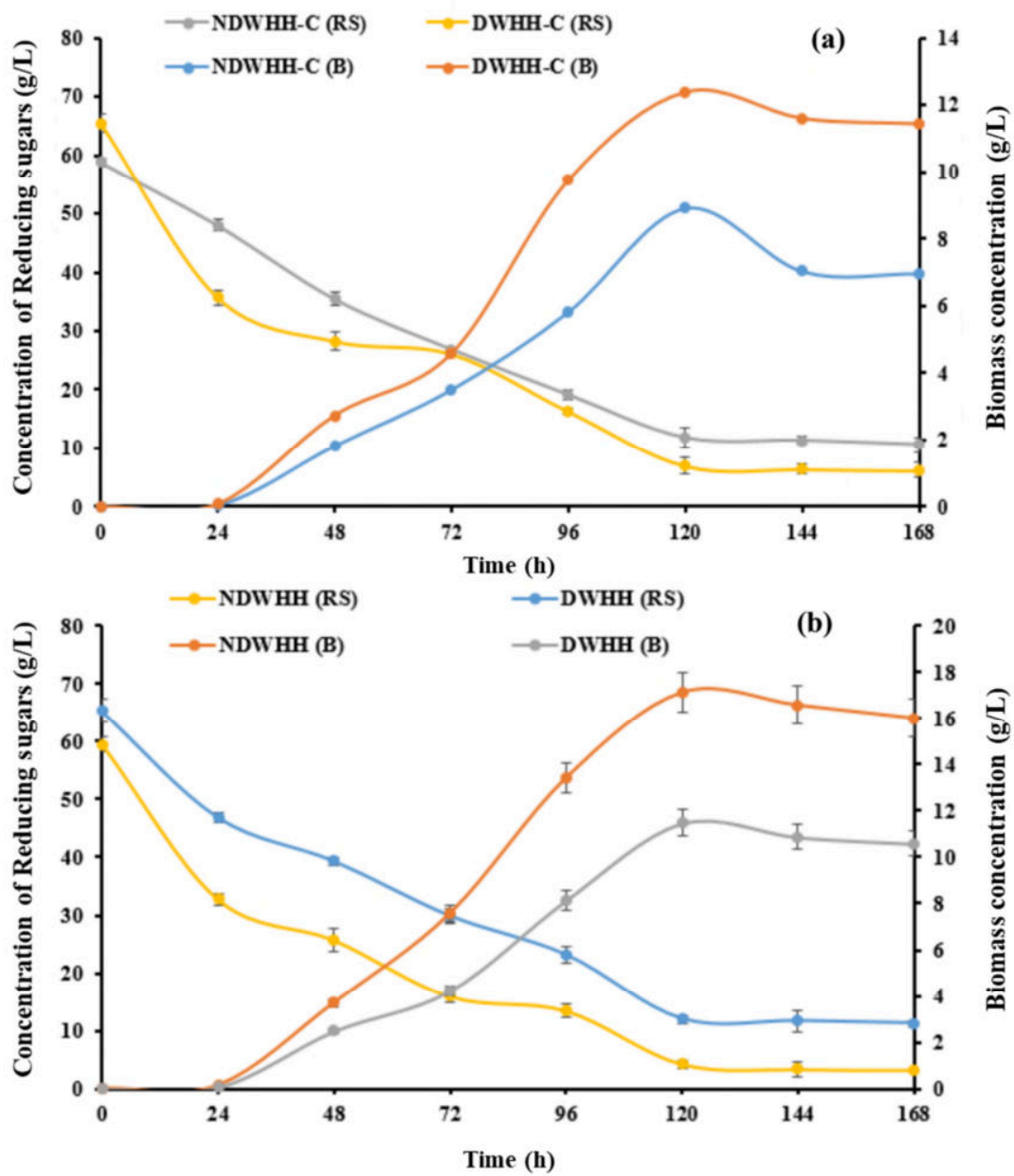


Fig. 1 Growth profile in terms of biomass concentration and reducing sugar consumption (a) NDWHH-C and DWHH-C; (b) NDWHH and DWHH

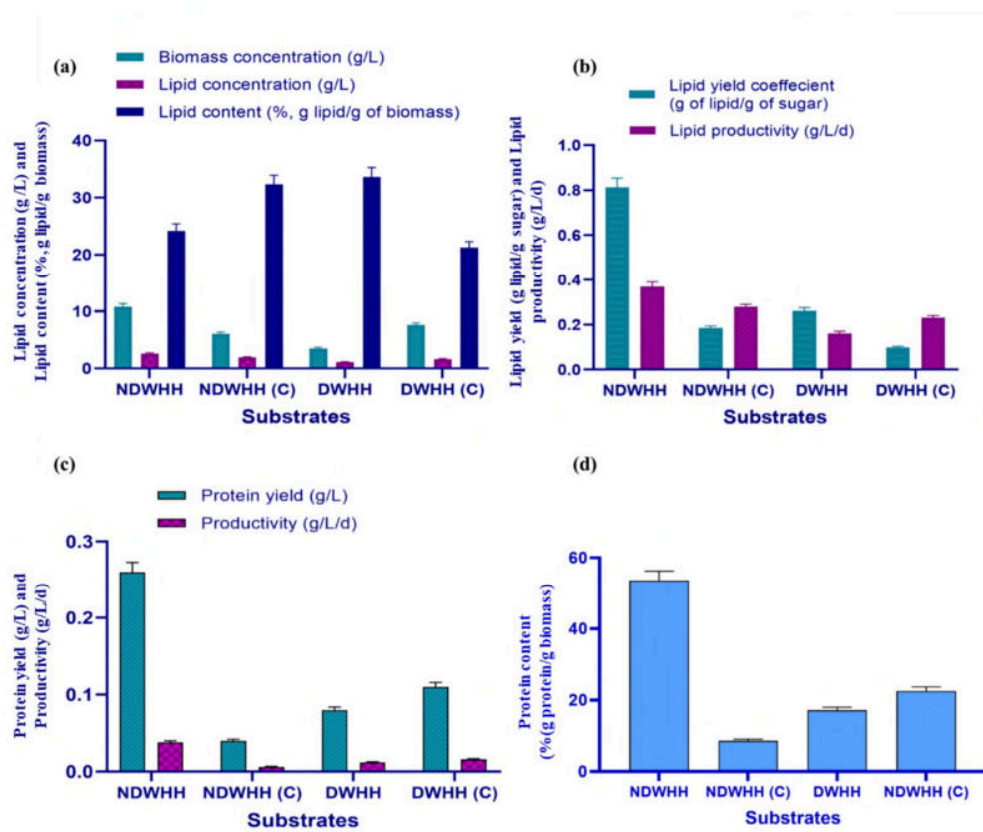


Fig. 2 Product yield analysis from *Rhodosporidium toruloides* (a) and (b) Lipid; (c) and (d) Protein

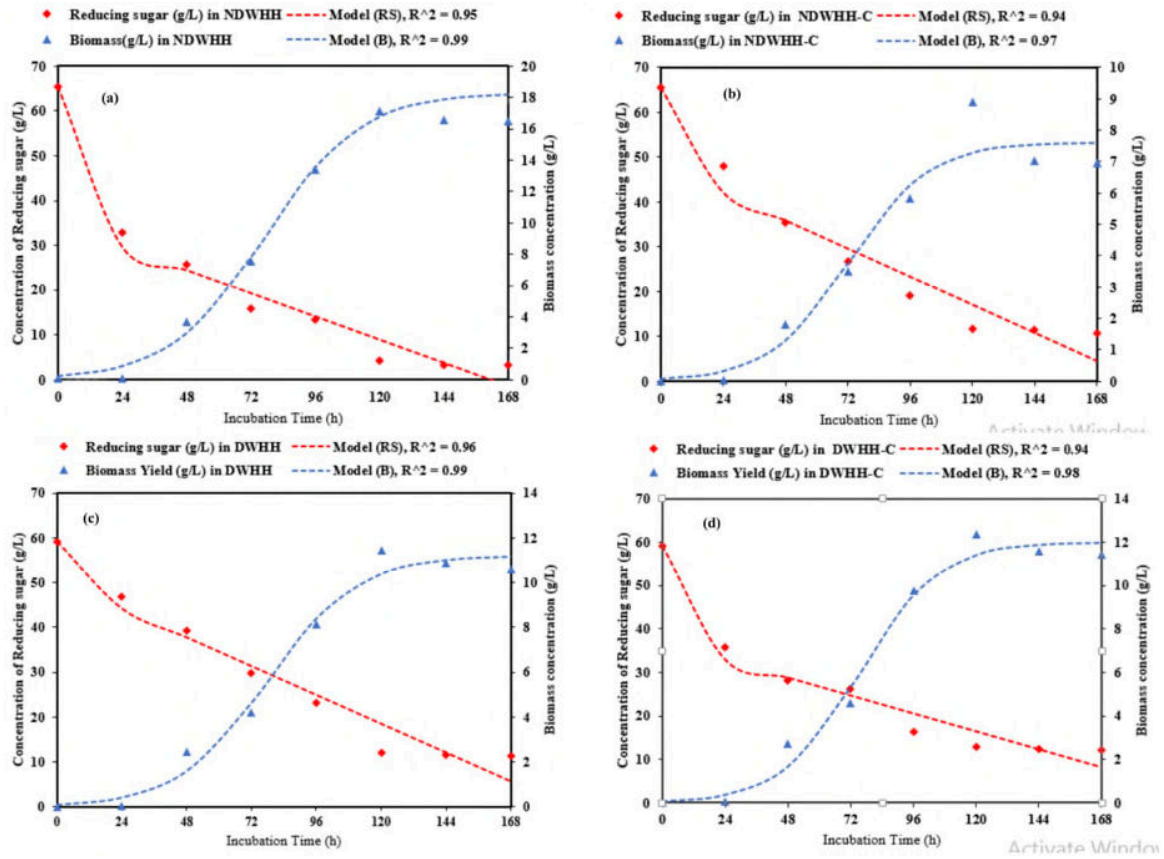


Fig. 3 Kinetic plots of (a) NDWHH; (b) NDWHH-C; (c) DWHH; (d) DWHH-C

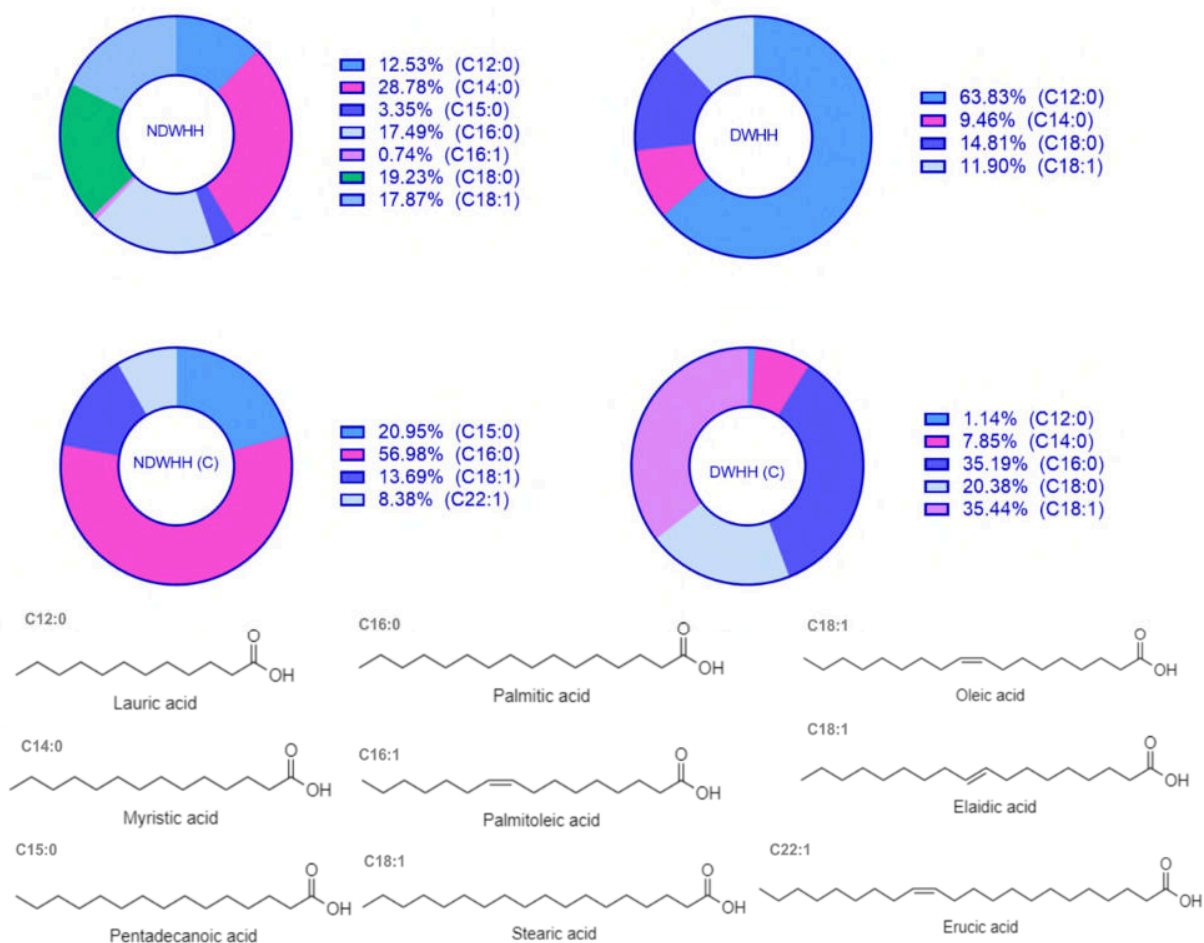
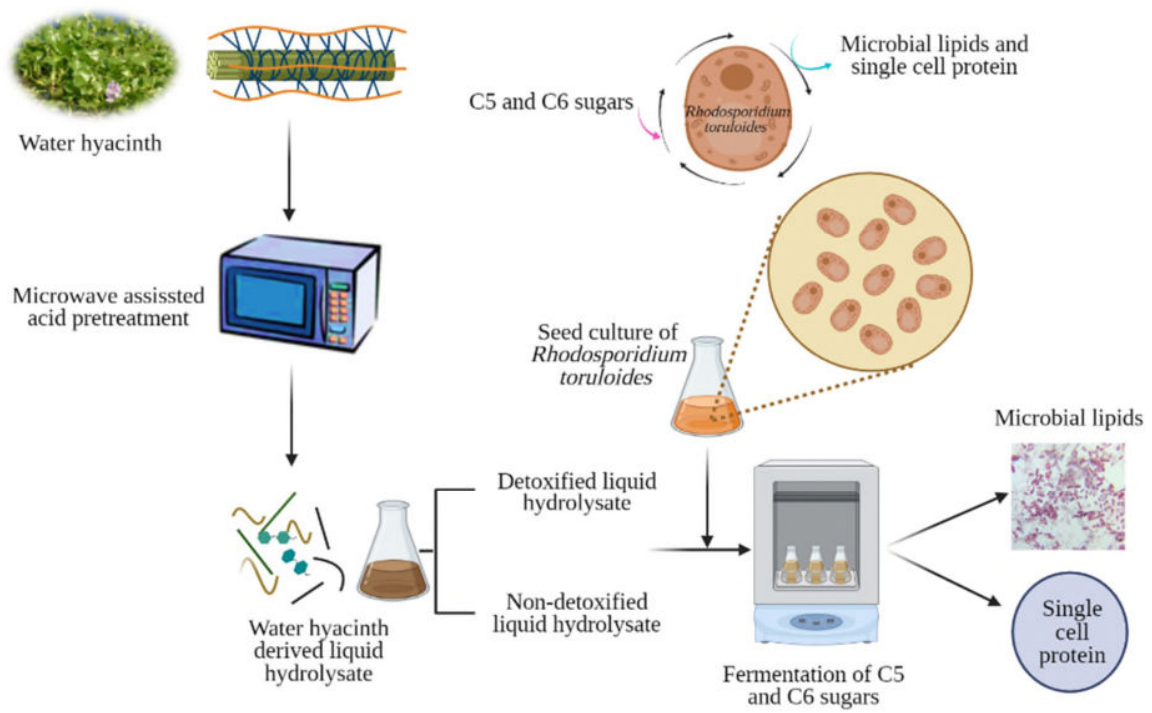


Fig. 4 Compositional analysis of lipids (based on GC-MS analysis) obtained through supplementation of different NDWHH and DWHH



Graphical abstract

**Bioprocessing of Fermentable Sugars Derived from Water Hyacinth into Microbial Lipids and Single Cell
Proteins by Oleaginous Yeast *Rhodospiridium toruloides* NCIM 3547**

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Supplementary Information

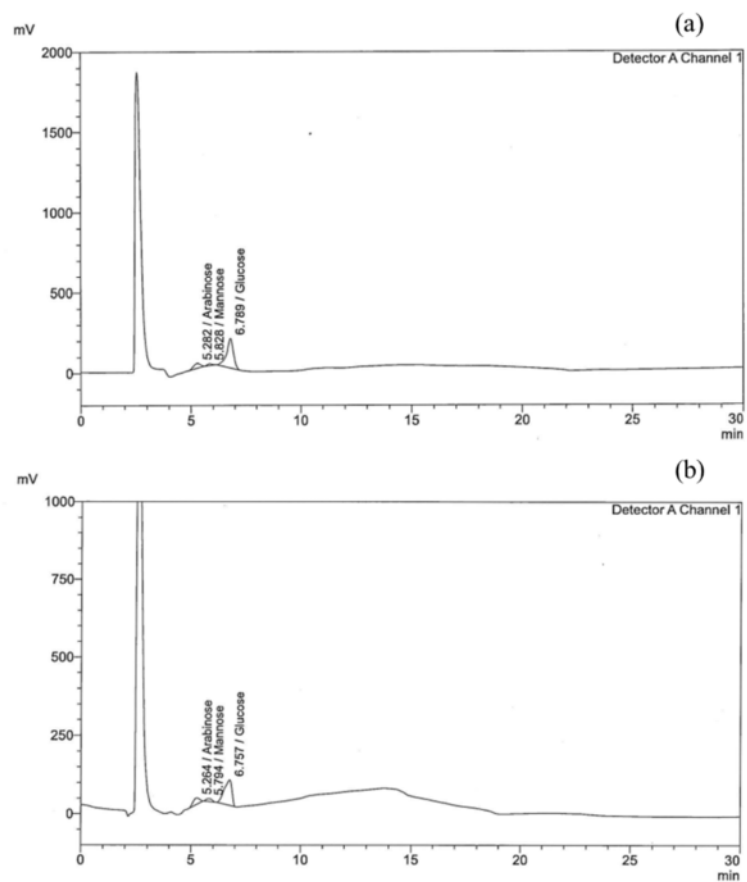


Fig. S3 HPLC chromatogram of NDWHH (a) and DWHH (b) for fermentable sugar analysis

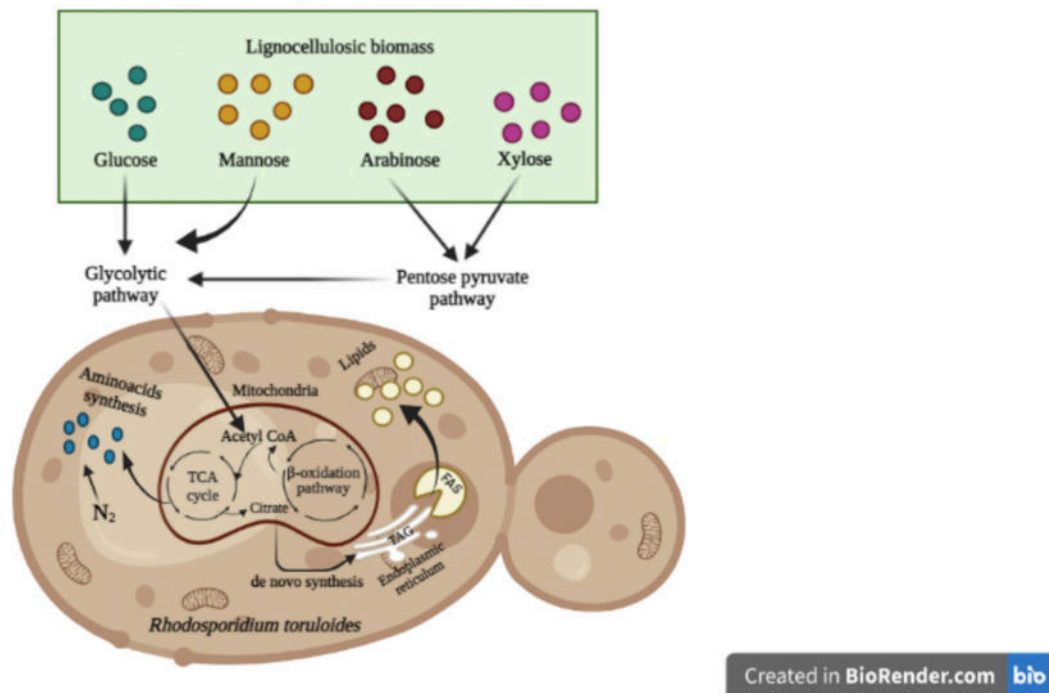


Fig. S4 Metabolic pathway for parallel synthesis of lipids and proteins in *Rhodosporidium toruloides*

Table S1. Fatty acid composition analysis from the GC-MS profile of the lipids obtained from the fermentation strategies

	Fatty acid	Type of fatty acid	Relative composition by area %			
			NDWHH	NDWHH-C	DWHH	DWHH-C
(C12:0)	Lauric acid	Saturated fatty acid				
	Dodecanoic acid		0.07	-	-	-
	Dodecanoic acid, 1,2,3-propanetriyl ester		0.21	-	14.5	-
	Dodecanoic acid, ethenyl ester		0.73	-	-	0.08
	Dodecanoic acid, 1-methylethyl ester		-	-	0.39	-
	Carbonic acid, nonyl vinyl ester		-	-	0.02	0.01
(C14:0)	Myristic acid	Saturated fatty acid				
	Myristic acid vinyl ester		2.32	-	2.21	0.62
(C15:0)	Pentadecanoic acid	Saturated fatty acid				
	Pentadecanoic acid, 14-methyl-, methylester		-	0.11	-	-
	Pentadecanoic acid, methyl ester		-	0.30	-	-
	Pentanoic acid, 4-methyl-4-nitro-, methyl ester		-	0.12	-	-
	Undec-10-ynoic acid, butyl ester		0.27	0.22	-	-
(C16:0)	Palmitic acid	Saturated fatty acid				
	Hexadecanoic acid, methyl ester		0.28	0.96	-	0.57
	Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester		1.13	0.41	-	-
	Hexadecanoic acid, ethyl ester		-	0.31	-	-
	Palmitic acid vinyl ester		-	-	-	0.51
						1.70

	Hexadecanoic acid		-	0.36	-	-
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester					
(C16:1)	Palmitoleic acid 9-hexadecenoic acid, methyl ester	Monounsaturated fatty acid	0.04	-	-	-
	Cyclohexanecarboxylic acid, phenylmethyl ester		0.02	-	-	-
(C18:0)	Stearic acid	Saturated fatty acid				
	Octadecanoic acid, 2-oxo-, methyl ester		0.84	-	-	1.38
	Octadecanoic acid, methyl ester		-	-	0.25	0.23
	Octadecanoic acid, octadecyl ester		-	-	3.21	-
	Octadecanoic acid		0.71	-	-	-
(C18:1)	Oleic acid 9-octadecenoic acid (z)-, methyl ester	Monounsaturated fatty acid	0.28	-	0.84	-
	Cis-9-octadecenoic acid, propyl ester		0.04	-	-	-
	9-octadecenoic acid (z)-, tetradecyl ester		-	0.13	-	-
	9-octadecenoic acid (z)-, tetradecyl ester		-	-	0.39	-
	Octadecanedioic acid		0.03	-	-	-
	Methyl 9-octadecenoate		0.20			2
(C22:1)	Erucic acid(c22:1) 13-docosenoic acid, methyl ester	Monounsaturated fatty acid	-	0.30	-	-
(C18:1)	Elaidic acid (e)-9-octadecenoic acid ethyl ester	Unsaturated fatty acid	0.89	0.36	1.55	0.80

Table S2 Concentration of inhibitors before and after detoxification of WH hydrolysate

Compound name	Inhibitor concentration before detoxification (µg/mL)	Inhibitors concentration after detoxification (µg/mL)	Fold decrease
	NDWHH	DWHH	
Furfural	3.87	1.74	2.22
5-HMF	7.05	5.15	1.36
Syringaldehyde	1.60	0.11	14.54

Table S3 Comparison of lipid accumulation using different lignocellulosic sugars including WH along with *R. toruloides* and other oleaginous microbes

Lignocellulosic biomass	Oleaginous microbes	Lipid production (g/L)	Reference
Rice straw hydrolysate	<i>Trichosporon fermentans</i>	11.5	Huang et al [32]
Wheat straw acid hydrolysate	<i>Cryptococcus curvatus</i>	5.8	Yu et al [33]
Carop pulp syrup	<i>Rhodospiridium toruloides</i> NCYC 921	0.91	Freitas et al [84]
Starch wastewater	<i>Rhodotorula glutinis</i>	14	Xue et al. [85]
Cassava starch	<i>R. toruloides</i> 21167	13.04	Gen et al. [86]
Aqueous extract of Cassia fistula L. fruit pulp	<i>Rhodospiridium kratochvilovae</i> HIMPA1	4.73	Patel et al. [87]
Switchgrass hydrolysate	<i>Y. lipolytica</i> YB-420	5.1	Quarterman et al. [82]
Saw dust (beechwood)	<i>Metschnikowia pulcherrima</i>	3.12	Longanesi et al. [58]
Saw dust (oak)	<i>Metschnikowia pulcherrima</i>	2.82	
Water hyacinth	<i>Cutaneotrichosporon oleaginosum</i>	7.3	Zhou et al. [81]
Corn cob hydrolysate	<i>Rhodotorula glutinis</i> NCIM 3168	3.71	Rane et al. [83]
Water hyacinth hydrolysate	<i>R. toruloides</i> NCIM 3547	2.62	This study



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