Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts

Liu, Zhijia; Feist, Adam; Dragone, Giuliano; Mussatto, Solange I.

Published in:
Journal of Cleaner Production

Link to article, DOI:
[10.1016/j.jclepro.2019.119308](https://doi.org/10.1016/j.jclepro.2019.119308)

Publication date:
2020

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Liu, Z., Feist, A., Dragone, G., & Mussatto, S. I. (2020). Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts. *Journal of Cleaner Production*, 249, [119308].
<https://doi.org/10.1016/j.jclepro.2019.119308>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts

Zhijia Liu, Adam M. Feist, Giuliano Dragone, Solange I. Mussatto

PII: S0959-6526(19)34178-2
DOI: <https://doi.org/10.1016/j.jclepro.2019.119308>
Reference: JCLP 119308
To appear in: *Journal of Cleaner Production*
Received Date: 14 July 2019
Accepted Date: 12 November 2019

Please cite this article as: Zhijia Liu, Adam M. Feist, Giuliano Dragone, Solange I. Mussatto, Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts, *Journal of Cleaner Production* (2019), <https://doi.org/10.1016/j.jclepro.2019.119308>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts

Zhijia Liu ^a, Adam M. Feist ^{a,b}, Giuliano Dragone ^a, Solange I. Mussatto ^{a,*}

^a Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, 2800, Kongens Lyngby, Denmark

^b Department of Bioengineering, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0412, USA

*Corresponding author. E-mail addresses: smussatto@biosustain.dtu.dk

Declarations of interest: none

ABSTRACT

This work aimed to support the development of a new process for the production of lipids and carotenoids by oleaginous yeasts using both cellulosic and hemicellulosic fractions of biomass as feedstock for fermentation. The concept behind this idea is that this strategy could lead to a sustainable pathway to produce these valuable components with potential to reduce the production costs. For the experiments, six oleaginous yeasts were initially cultivated in synthetic media containing glucose or xylose as carbon sources and different carbon to nitrogen ratios in order to identify the top producers. *Rhodospiridium toruloides* NRRL Y-1091 and *Lipomyces starkeyi* NRRL Y-1389 produced the highest amount of carotenoids and lipids, respectively. Then, the performance of these two strains when cultivated in wheat straw cellulosic and hemicellulosic hydrolysates was assessed. Detoxification of the cellulosic and hemicellulosic hydrolysates with activated charcoal removed 87.80% and 77.17% of inhibitors, respectively, and significantly improved the performance of the two yeasts. The highest concentration of lipids, 3.99 ± 0.35 g/L, was obtained by cultivating *L. starkeyi* in washed cellulosic hydrolysate; while the highest production of carotenoids, 24.58 ± 1.88 mg/L, was obtained by cultivating *R. toruloides* in decolorized cellulosic hydrolysate. Increasing the tolerance of oleaginous yeasts to inhibitors resulting from biomass pretreatment and/or using cost-effective detoxification methods were identified as key parameters for further development of this process at commercial scale.

Keywords: Oleaginous yeasts; Lipids; Carotenoids; Wheat straw hydrolysate; Fermentation

Abbreviation list

CH: Raw cellulosic hydrolysate

DCH: Decolorized cellulosic hydrolysate

CCH: Concentrated cellulosic hydrolysate

CDCH: Concentrated and decolorized cellulosic hydrolysate

WCH: Washed cellulosic hydrolysate

WCCH: Washed and concentrated cellulosic hydrolysate

SCM: Synthetic concentrated medium

HH: Hemicellulosic hydrolysate (after pretreatment)

PH: Post hydrolyzed hemicellulosic hydrolysate

C2H: Post hydrolyzed and 2-fold concentrated hemicellulosic hydrolysate

C2DH: Post hydrolyzed, 2-fold concentrated and decolorized hemicellulose hydrolysate

C4H: Post hydrolyzed and 4-fold concentrated hemicellulosic hydrolysate

C4DH: Post hydrolyzed, 4-fold concentrated and decolorized hemicellulosic hydrolysate

1. Introduction

Biodiesel has been drawing increased attention, as it is renewable, nontoxic, biodegradable, suitable for sensitive environments, and can be used in most diesel engines with no or only minor modifications (Alleman et al., 2016). In addition, utilizing biodiesel to displace petroleum-derived diesel fuel can reduce 60% carbon dioxide emissions (Chen et al., 2009). However, at present, biodiesel is mainly produced from transesterification of vegetable oils, animal fats and recycled cooking greases or oil (Alleman et al., 2016). These raw materials can pose a competition for

food production and have the risk of an unstable supply. Single cell oil produced by oleaginous microorganisms have the potential to be used as feedstock for the production of various fatty acids derived compounds with a wide range of applications (Vasconcelos et al., 2019). Compared to other microorganisms, oleaginous yeasts can accumulate up to 70% of lipids (dry cell mass basis) (Probst et al., 2016) with fast growth rates and high cell densities under nutrient deprivation conditions. Therefore, the production of lipids by such yeasts is expected to become a sustainable and significant source of biodiesel in the future. In addition, some oleaginous yeasts can accumulate carotenoids simultaneously with lipids. Carotenoids are valuable natural pigments, and normally act as vitamin A precursors. Moreover, due to their coloring, antioxidant and possible tumor-inhibiting activity, carotenoids have been widely used in pharmaceutical, chemical, food and feed industries (Frengova and Beshkova, 2009). Extraction from vegetables and chemical synthesis are currently the main ways to obtain commercial carotenoids. However, due to seasonal and geographic limitations for vegetable extraction and hazardous waste generated in chemical synthesis, microbial carotenoids produced from cheaper carbon sources are becoming appealing (Mata-Gómez et al., 2014). At present, there is no commercial production of microbial lipid and carotenoid; thus, more efforts should be made to promote their industrialization.

Another merit of oleaginous yeasts is that they can metabolize different carbon sources, including hexose and pentose sugars. Various types of waste and low-cost carbon sources, including food processing wastewater, have been suggested as feedstock to produce microbial lipids by oleaginous yeasts and a significant pollution removal from these wastewaters has been observed (Arous et al., 2016). Lignocellulosic biomass is another attractive feedstock, since it is abundant, inexpensive and renewable.

Cellulose and hemicellulose, the polymers of glucose and xylose, respectively, are important components of lignocellulosic materials and cannot be directly utilized by yeasts. Fermentable sugars need to be released from these fractions by pretreatment and hydrolysis of biomass. However, some toxic compounds derived from the degradation of cellulose, hemicellulose and lignin are also obtained at this stage.

Hydroxymethylfurfural, furfural and acetic acid are the most common inhibitors from degradation of cellulose and hemicellulose, respectively; while vanillin, vanillic acid, 4-hydroxybenzoic acid, syringaldehyde are typical lignin degradation compounds (Fan et al., 2018). The poor endurance of yeasts to these inhibitors is an important barrier for using lignocellulosic biomass in a commercial scale. These compounds not only inhibit yeast's growth but also affect the accumulation of lipids in oleaginous yeasts (Patel et al., 2016). Another barrier for efficient utilization of lignocellulosic biomass is that most of the industrial microorganisms are unable to assimilate pentose sugars, which are the main carbon sources in hemicellulosic hydrolysates.

Some attempts have been made to assess the performance of oleaginous yeasts in lignocellulosic hydrolysates. In a recent study, *Rhodotorula glutinis*, *Rhodospiridium toruloides*, *Saitoella coloradoensis*, *Lipomyces starkeyi* and *Yarrowia lipolytica* have been reported as promising strains to utilize diluted corn stover and switchgrass hydrolysates (Slininger et al., 2016). Other attempts included the cultivation of *L. starkeyi* in diluted birch wood hemicellulosic hydrolysate (Brandenburg et al., 2016).

A study on efficient utilization of both cellulosic and hemicellulosic fractions to produce microbial lipids and carotenoids could predict the production performance in industrial scale and generate knowledge to assess the economic and environmental benefits of a commercial production. Based on it, the present study was designed to

bridge this gap providing new data and information relevant for the development of a new process for the production of lipids and carotenoids at industrial scale. Briefly, six oleaginous yeasts, including *R. glutinis*, *R. toruloides*, *S. coloradoensis*, *L. starkeyi* and 2 different strains of *Y. lipolytica* were tested in glucose or xylose media with carbon to nitrogen ratios ranging from 25 to 100 in order to identify the best lipid and carotenoid producers. The best candidates were then cultivated in cellulosic and hemicellulosic hydrolysates obtained from wheat straw and their performances were compared. Then, the hydrolysates were concentrated and detoxified in an attempt to improve the performance of the selected yeasts. Finally, the implication of our study on an industrial production scale was also discussed.

2. Materials and methods

2.1. Microorganisms and inoculum preparation

Oleaginous yeasts *R. glutinis* NRRL Y-2502, *R. toruloides* NRRL Y-1091, *S. coloradoensis* NRRL YB-2330, *L. starkeyi* NRRL Y-1389, *Y. lipolytica* NRRL YB-392, and *Y. lipolytica* NRRL YB-567 were obtained from the Agricultural Research Culture Collection, USA. Yeasts were maintained in YPD agar plates at 4 °C, being transferred to new plates once a month. In the pre-cultivation step, one loop of yeast cell was transferred to 100 mL YPD media in 250 mL flasks and cultivated at 30 °C, 250 rpm for 24 h. The pre-culture was then separated by centrifugation (5000 rpm, 5 min) and inoculated to the synthetic screening media.

2.2. Yeast growth in synthetic media with different carbon to nitrogen (C:N) ratios

Yeasts were cultivated synthetic media containing glucose or xylose in order to compare their growth and ability to accumulate products from different carbon sources. Synthetic media contained 50 g/L glucose or xylose, 1.7 g/L yeast nitrogen base without amino acid, and ammonium sulfate (Sigma-Aldrich, Denmark). Different amount of ammonium chloride as nitrogen source was added to control the C:N ratio. C:N ratio was calculated as the ratio of moles of carbon available in sugars (glucose or xylose) to the moles of nitrogen (ammonium chloride). The pH of screening media was around 4.5. All flasks were incubated at 30 °C, 250 rpm for 120 h. Samples were taken every 24 h to determine yeast cell mass. After fermentation, lipids and carotenoids were also determined.

2.3. Preparation of wheat straw hydrolysates

Wheat straw was provided by the Danish Technological Institute (Denmark). Dried wheat straw was milled to a size of about 180 - 1800 µm and pretreated by hydrothermal pretreatment. Briefly, 30 g wheat straw (dry matter) was mixed with 300 mL distilled water in a 600 mL non-stirred pressure vessel (Parr Series 4760, Parr Instrument Company, Moline, IL) and held at 195 °C in a silicone oil bath (Lauda Eco Gold) for 45 min. After pretreatment, the pressure vessel was placed in an ice bath until reaching ambient temperature and the solid and liquid fractions were then separated (**Figure 1**).

The liquid fraction (hemicellulosic hydrolysate) was post-hydrolyzed with 2% sulfuric acid (w/w) in a pre-heated autoclave at 121 °C for 20 min. The reaction was

stopped by cooling with ice. Then, the pH was neutralized by the addition of CaCO_3 , and the precipitate was removed by centrifugation at 10,000 rpm for 20 min.

The solid fraction (with about 76% moisture) used for production of cellulosic hydrolysates was divided into 2 parts. One part was directly used for enzymatic hydrolysis using an enzyme loading of 12.25 FPU/g dry pretreated wheat straw. For the experiments, the pretreated wheat straw (unwashed) was mixed with a cellulase complex (Cellic C-Tec2, Novozymes – Denmark) in 0.05 M sodium citric buffer pH 5.8. The final pH of the mixture was 5.3 as the solid material contained some acid coming from pretreatment, which lowered the final pH of the mixture to 5.3. The enzymatic hydrolysis was conducted in an incubator equipped a bottle roller at 50 °C, 50 rpm for 72 h. The other solid part was washed with distilled water until °Brix (total soluble solids in aqueous solution) and pH reached 0 and 7, respectively. The washed wheat straw was hydrolyzed by cellulase at the same conditions mentioned above. In this case, the pH of the buffer was adjusted to 5.3, since acid was removed by washing of the solid material and did not affect the final pH of the mixture. After that, the remaining solid residue and hydrolysate were separated by centrifugation at 10,000 rpm, 20 min. Concentration of hemicellulosic and cellulosic hydrolysates was carried out in a rotary evaporator (RV 10 control, IKA) at 50 °C, 10 rpm, 72 mbar and condensation at 5 °C. Decolorization of hydrolysates was conducted by adding 2% (w/w) activated charcoal (Sigma- Aldrich, Denmark) and stirring magnetically for 30 min at room temperature. Used charcoal was removed by centrifugation (10,000 rpm, 20 min). The obtained liquids were sterilized by 0.22 μm membrane filtration and used for yeast cultivation.

2.4. Preparation of concentrated synthetic media

To study the influence of high sugar concentration on yeast growth, synthetic media with the same sugar concentration as that found in washed and concentrated cellulosic hydrolysate were used. Yeast nitrogen base without amino acid and ammonium sulfate (Sigma-Aldrich, Denmark) were added to provide necessary nutrients for yeasts growth. Ammonium chloride was the nitrogen source used to control C:N ratio to 100, as described above.

2.5. Yeast cultivation in hemicellulosic and cellulosic hydrolysates

The oleaginous yeasts that produced the highest concentration of lipids and carotenoids in synthetic media were cultivated in hemicellulosic and cellulosic hydrolysates at 30 °C, 250 rpm for 120 h. Inoculum concentration was 1 g/L. Samples were harvested every 24 h, centrifuged and washed twice with distilled water. Fermentation supernatant was used for sugars analysis, while the cells were used for determination of biomass concentration, total lipid and carotenoid quantification.

2.6. Analyses

Sugars, acetic acid, hydroxymethylfurfural (HMF) and furfural concentrations were determined using a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germany) equipped with an Aminex HPX-87H column (300×7.8 mm, Bio-Rad, USA) eluted with 0.005 M H₂SO₄, at 60 °C with 0.6 mL/min flow rate for 50 min. Phenolic compounds were analyzed by colorimetric method as described by Ballesteros et al. (2014).

Lipids concentration was quantified by sulfo-phospho-vanillin colorimetric assay (Izard and Limberger, 2003) adapted to a 96-well microplate for absorbance reading. Sunflower seeds oil (Sigma-Aldrich, Denmark) dissolved in chloroform was used as calibration standard. Dimethylsulfoxide (DMSO) was used to extract and dissolve carotenoids for quantification of carotene concentration. Briefly, cells from 1 mL fermentation broth were harvested by centrifugation in 2 mL tube and washed twice. Then, 1 mL DMSO was added and the mixture was vortexed at 30 °C, 2000 rpm for 15 min. Extractives were collected by centrifugation at 10,000 rpm and 5 min. The extraction was repeated several times until cells became white. All extractives were combined and transferred to 96 well-plates. A microplate reader (BioTek Synergy Mx) was used to read extracted samples with full spectrum scan (230-900 nm) at 5 nm interval.

The concentration of carotenoids was measured by the absorbance of orange solvent at 450 nm against DMSO. β -carotene dissolved in DMSO was used as standard.

To analyze the lipid composition, the fermentation broth was centrifuged at 1000 rpm for 5 min. The resultant pellet was washed twice and then suspended in distilled water. The same volume of chloroform: methanol (2:1, v/v) mixture was added and vortexed during 15 min to extract lipids. The organic and water phases were separated by centrifugation (10,000 rpm, 5 min). The bottom phase (chloroform phase) was removed and dried under nitrogen atmosphere. The extracted lipids were converted to fatty acid methyl esters and then analyzed by an Agilent 7890A GC system equipped with a column X from Supelco according to the AOCS official methods (The American Oil Chemists' Society, 1998a; The American Oil Chemists' Society, 1998b).

2.7. Accumulation, yield and productivity determinations

The accumulation of lipids and carotenoids was defined as the ratio of lipids or carotenoids produced to dry cell mass. Lipid accumulation was expressed as %, and carotenoid accumulation was expressed as mg/g. The yield of products refers to the amount of product generated divided by the amount of carbon source consumed. Lipid yield was expressed as g/g carbon, and carotenoid yield was expressed as mg/g carbon. Productivity was defined as the concentration of product generated per time of fermentation. Both lipid and carotenoid productivity were expressed as mg/L h.

3. Results and discussion

3.1. Yeasts performance in synthetic media under different C:N ratios

The six different oleaginous yeasts were initially cultivated in synthetic media containing glucose or xylose as sole carbon sources in order to compare their abilities to utilize both types of sugars. As oleaginous yeasts can switch their metabolism to lipid accumulation under nutrient starving conditions (Beopoulos et al., 2011), lipid and carotenoid production ability was measured and compared under different carbon to nitrogen ratios. Results showed that only *R. toruloides*, *S. coloradoensis* and *L. starkeyi* were able to metabolize both glucose and xylose as carbon sources for cell growth (**Table 1**). Among them, *L. starkeyi* presented the best growth performance in terms of final cell density, reaching a cell mass concentration of 11.73 ± 0.18 g/L in glucose media and 12.17 ± 0.08 g/L in xylose media, after 120 h. Alternatively, *R. glutinis* showed the lowest cell mass concentration (1.02 ± 0.19 g/L) in glucose media at the end of cultivation.

Lipid and carotenoid production varied to the different yeasts and C:N ratios. The highest lipid concentration (3.61 ± 0.40 g/L) was achieved by *L. starkeyi* cultivated in xylose media with a C:N ratio of 50. It was also found that *Y. lipolytica* (YB-392) presented the maximum lipid accumulation ($39.51 \pm 1.36\%$) when cultivated in glucose medium with a C:N ratio of 100, followed by *Y. lipolytica* (YB-567) ($36.39 \pm 2.08\%$) at the same cultivation conditions. However, both yeasts were unable to use xylose as carbon source (**Table 1**).

Results from full wavelength scan of yeast extracts showed that only *R. glutinis*, *R. toruloides* and *S. coloradoensis* were able to produce carotenoids (data not shown). Most carotenoids have the maximum absorption under wavelength between 400 to 550 nm (Rodriguez-amaya, 2001). The highest carotenoid concentration (9.23 ± 0.97 mg/L) was obtained by *R. toruloides* when using xylose as carbon source and a C:N ratio of 50. This yeast also presented the highest carotenoid accumulation (2.99 ± 0.66 mg/g) in xylose media with a C:N ratio of 25. Based on the above, *L. starkeyi* and *R. toruloides* were selected as the best producers of lipids and carotenoids, respectively. It is worth noting that no significant differences in cell mass and lipid concentration of *L. starkeyi* were found when cultivated in glucose or xylose media with different C:N ratios, which may indicate that this yeast is not influenced by the ratio in the studied range (25 to 100). Alternatively, lipid concentration of *R. toruloides*, *Y. lipolytica* (YB-392) and *Y. lipolytica* (YB-567) increased with higher C:N ratios.

3.2. Fatty acids composition

The major fatty acids produced by the six oleaginous yeasts were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2), which

accounted for more than 50% of the total fatty acids produced. **Table 2** lists the main fatty acids produced by these yeasts in synthetic media with different C:N ratios. The most abundant fatty acids produced by *R. glutinis*, *L. starkeyi*, *Y. lipolytica* (Y-392) and *Y. lipolytica* (Y-567) were oleic acid (C18:1), followed by palmitic acid (C16:0) and linoleic acid (C18:2), which was consistent with previous studies (Yu et al., 2011). In addition, only *R. glutinis* was able to produce about 6.80% of C18:3 (n-6) (data not shown in Table 2), which may indicate that only *R. glutinis* contains an active $\Delta 6$ desaturase. It is worthy to notice that the percentage of C18:1 in *R. toruloides* increased as the C:N ratio increased. Unlike the other oleaginous yeasts, linoleic acid was the predominant fatty acid found in *S. coloradoensis*. Fatty acids with 16 to 18 carbon atoms are optimal feedstock for biodiesel production due to similar fuel properties (Steen et al., 2010).

The different oleaginous yeasts tested in this study produced similar fatty acids due to common lipid biosynthetic pathways and the presence of similar enzyme complex. In oleaginous yeasts, acetyl-CoA and malonyl-CoA, the building blocks of lipid biosynthesis, are catalyzed by type I fatty acid synthases to form saturated acyl chains with 16 (C16:0) or 18 (C18:0) carbon atoms (Qadeer et al., 2017). These acyl chains are modified by desaturases to form unsaturated C16 and C18 fatty acids. The percentage of fatty acids differs among the different species and for the different synthetic media.

3.3. Composition of cellulosic hydrolysates

The composition of the different cellulosic hydrolysates produced from wheat straw is shown in **Table 3**. The concentration of furfural and phenolic compounds in the

raw cellulosic hydrolysate (CH) were 0.19 ± 0.01 g/L and 1.45 ± 0.07 g/L, respectively. After the detoxification step, it was found that using either activated charcoal for obtaining decolorized cellulosic hydrolysate (DCH) or washing the pretreated wheat straw for production of washed cellulosic hydrolysate (WCH), was effective to remove furfural and phenolic compounds. Activated charcoal removed 87.80% of inhibitors while biomass washing removed 77.17% of inhibitors. Activated charcoal adsorption is a cost effective and efficient method for removal of inhibitors compounds from biomass hydrolysates (Mussatto et al., 2005). The high degree of porosity, extensive surface area, and high degree of surface reactivity allows activated charcoal to absorb small molecule compounds in liquids (Ansari and Mohammad-Khah, 2009; Mussatto et al., 2010). Biomass washing is another operation for production of clarified hydrolysates, widely used in commercial-scale ethanol production from lignocellulosic materials (Larsen et al., 2012). Since these inhibitors are water-soluble compounds generated during pretreatment, washing can remove these compounds keeping the insoluble structure unaltered. It is worth noting that the efficiency of saccharification of the washed wheat straw ($81.99 \pm 0.21\%$) was higher than that of unwashed wheat straw ($74.01 \pm 0.80\%$). According to Qin et al. (2016), phenolic compounds can inactivate cellulases and inhibit enzymatic hydrolysis. However, when compared to biomass washing, activated charcoal can work in the detoxification of both, cellulosic and hemicellulosic hydrolysates. After detoxification, the used charcoal can be easily removed by filtration. Moreover, according to Gupta et al (2016), used activated charcoal can be recycled after regeneration.

When the raw cellulosic hydrolysate was concentrated 2-fold (CCH), the concentration of acetic acid increased to 2.15 ± 0.04 g/L. Although acetic acid (≥ 5 g/L)

has an inhibitory effect on the growth of oleaginous yeasts, it can also be metabolized as a carbon source by these microorganisms when present in lower concentrations (Chen et al., 2009). Alternatively, the furfural concentration was reduced from 0.19 ± 0.01 g/L in CH to 0.02 ± 0.00 g/L in CCH. In fact, it has been reported that the concentration of hydrolysates at 50 °C under vacuum condition results in removal of furfural since this compound is volatile under these conditions (Mussatto and Roberto, 2004).

3.4. Composition of hemicellulosic hydrolysates

Hydrothermal pretreatment can overcome the recalcitrance of lignocellulosic biomass by depolymerizing hemicellulose. However, the main carbohydrates released by this pretreatment to the liquid fraction are xylooligosaccharides that cannot be metabolized by yeast. Therefore, a post hydrolysis of xylooligosaccharides in the liquid fraction must be performed in order to increase xylose concentration in hemicellulosic hydrolysates. The concentration of xylose in the hemicellulosic hydrolysates obtained in this study after pretreatment of wheat straw and further post hydrolysis of the liquid fraction (PH) was 14.20 ± 0.04 g/L (**Table 4**).

When the post hydrolyzed hemicellulosic hydrolysate (PH) was concentrated 2-fold (C2H), the concentration of xylose and acetic acid increased to 24.76 ± 0.16 g/L and 3.67 ± 0.05 g/L, respectively, while that of furfural was significantly reduced to 0.07 ± 0.01 g/L (**Table 4**). After decolorization of C2H with activated charcoal (C2DH), the concentration of phenolic compounds decreased from 2.52 ± 0.06 g/L to 0.63 ± 0.14 g/L, representing a reduction of 75% of inhibitors. Alternatively, the 4-fold concentration and decolorization of C2H (C4DH) resulted in 51.63 ± 0.20 g/L xylose, 4.86 ± 0.11 g/L acetic acid and 0.83 ± 0.03 g/L phenolic compounds. These results

demonstrate that the concentration of main inhibitors, especially phenolic compounds and furfural, was reduced to relative low levels by applying concentration and decolorization methods. Activated charcoal absorption can be then considered as a promising detoxification method to be used at industrial scale.

3.5. Fermentation of cellulosic hydrolysates by selected yeasts

Fermentation assays revealed that the yeasts previously selected as best producers of lipids and carotenoids in synthetic media, *L. starkeyi* and *R. toruloides* respectively, were unable to grow in cellulosic hydrolysate (CH). Growth of these strains could have been affected by the presence of inhibitory compounds derived from lignocellulose degradation. The toxicity of phenolic compounds lies in the membrane permeation and increasing level of reactive oxygen species. The former can attenuate function of selective barriers and substrates exchanging of intercellular membrane of yeasts, and the latter causes cytoskeleton damage, DNA mutagenesis and programmed cell death (Wang et al., 2018).

The sugars consumption performance of *L. starkeyi* and *R. toruloides* was similar after removing furfural and lowering the concentration of phenolic compounds to 0.19 ± 0.02 g/L - DCH. When cultivated in DCH, both yeasts metabolized all carbon sources in 72 h (**Figure 2**). It is worth noting that xylose uptake started only after most glucose (> 60%) was consumed. The sequential consumption of sugars by oleaginous yeasts was in good accordance with previous study (Zhao et al., 2008) and has been attributed to glucose repression on xylose transport (Ounine et al., 1985). The highest biomass and lipid concentrations for *R. toruloides* were 17.95 g/L and 3.36 g/L (18.73% of dry cell mass), respectively, at 96 h. At that time, the highest carotenoid concentration (24.58

mg/L) was also obtained (**Figure 2A**). The maximum yield of cells, lipids and carotenoids for *R. toruloides* were 0.25 ± 0.02 g/g carbon, 0.05 ± 0.01 g/g carbon and 0.32 ± 0.03 mg/g carbon, respectively. When considering the growth of *L. starkeyi* in DCH, it was observed that although the highest cell mass concentration was obtained at the end of the experiment (120 h), the maximum lipid concentration (3.23 ± 0.22 g/L) was found at 96 h (**Figure 2B**).

Cultivation of *R. toruloides* and *L. starkeyi* in washed cellulosic hydrolysate (WCH) with a concentration of phenolic compounds of 0.42 ± 0.04 g/L, higher than that found in DCH (0.19 ± 0.02 g/L), resulted in different yeast's performance (**Figure 3**). The consumption of carbon sources by *R. toruloides* was negatively affected when WCH was used as fermentation medium. About 29% of initial glucose and 88% of initial xylose remained in the fermentation broth at the end of the fermentation (**Figure 3A**). Maximum cell mass, lipid and carotenoid concentrations were also lower than those obtained with DCH (**Figure 2A**). However, the use of WCH leads to higher accumulation and yield of lipids and carotenoids. It is probable that some nitrogen sources have been removed during the washing step of the solid fraction, which resulted in increased C:N ratio in WCH in comparison to that in DCH. The screening experiment performed in synthetic media (section 3.1) indicated that *R. toruloides* had a better performance under higher C:N ratio. Alternatively, fermentation parameters of *L. starkeyi* cultivated in WCH (**Figure 3B**) did not present a significant difference when compared to those obtained in DCH. These results were also in good agreement with those found during the screening experiments in synthetic media described above where fermentation parameters were not influenced by the C:N ratio in the range of 25 to 100.

Concentrated detoxified cellulosic hydrolysates (WCCH and CDCH) were also tested as culture media for the production of lipids and carotenoids by *R. toruloides* and *L. starkeyi*. Results showed that both yeasts were unable to grow in WCCH, probably due to synergistic effects between high sugar concentration and phenolic compounds. The use of a concentrated cellulosic hydrolysate (CDCH) with a content of phenolic compounds of 0.57 ± 0.03 g/L, lower than that found in WCCH (0.96 ± 0.03 g/L), enabled the cultivation of *R. toruloides* only. However, yeast growth was partially inhibited since biomass concentration increased up to 6.60 ± 0.67 g/L after 120 h of cultivation (**Figure 4A**). In addition, about 63% of initial glucose concentration remained in the fermentation broth at the end of the experiment. The synergistic effects of inhibitors (i.e., phenolic compounds and acetic acid) may have also been responsible for the complete inhibition of *L. starkeyi* growth in CDCH. It has been reported that cell growth and lipid production by oleaginous yeasts are inhibited in a greater extent by the synergistic effect of inhibitors because the minimum critical inhibitory concentration for each inhibitor is reduced (Hu et al., 2009). Further, Zhao et al. (2012) found that 2 g/L acetic acid can completely inhibit the growth of *L. starkeyi* when inhibitors from lignocellulosic hydrolysates are present in the fermentation broth, while a concentration of 20 g/L acetic acid is needed for a complete inhibition of yeast cell growth when no other inhibitors are present in the medium.

To investigate the effect of high sugar concentration on cell growth and production of lipids and carotenoids by *R. toruloides* and *L. starkeyi*, additional fermentation runs were carried out using synthetic medium without inhibitors and with the same sugar concentration as that found in the concentrated cellulosic hydrolysates. Although both yeasts were able to grow in the synthetic media, it was observed that *L.*

starkeyi (**Figure 4C**) showed better fermentation performance than *R. toruloides* (**Figure 4B**). The maximum cell mass and lipid concentration for *L. starkeyi* were 16.75 ± 0.28 g/L and 3.51 ± 0.37 g/L ($20.95 \pm 1.91\%$ accumulation), respectively, at 120 h. It is also worth noting that the use of concentrated and decolorized cellulosic hydrolysate (CDCH) leads to a higher cell mass concentration of *R. toruloides* (6.60 ± 0.67 g/L, **Figure 4A**) when compared to that obtained with synthetic media (4.50 ± 0.40 g/L, **Figure 4B**). This result suggests that lignocellulosic hydrolysates can provide additional nutrients for yeast growth. Previous studies have reported that *R. toruloides* could reach up to 25% higher biomass concentration when bagasse hydrolysate was used as carbon source in comparison to that obtained with synthetic media (Zhao et al., 2012).

3.6. Fermentation of hemicellulosic hydrolysates by selected yeasts

Different hemicellulosic hydrolysates were also tested as carbon sources for the production of lipids and carotenoids by *R. toruloides* and *L. starkeyi*. Results showed that both yeasts were able to grow in medium (C2DH) containing 0.63 ± 0.14 g/L of phenolic compounds. However, they presented different fermentation performances (**Figure 5**). Glucose and acetic acid, for example, were completely metabolized by *R. toruloides* within 24 h (**Figure 5A**), while 96 h were needed for the consumption of both carbon sources by *L. starkeyi* (**Figure 5B**). Furthermore, a long lag phase (72 h) was observed during the growth of *L. starkeyi* in C2DH.

A final comparison of the two strains revealed that, despite a sluggish onset of fermentation, cell mass and lipid productivity for *L. starkeyi* were in general higher than those obtained by *R. toruloides* at the end of the experiment (**Table 5**). In terms of hydrolysate medium, fermentation parameters such as accumulation, yield and

productivity of lipids resulting from *L. starkeyi* cultivation in C2DH, were lower than those obtained by this yeast in cellulosic hydrolysates (DCH and WCH, **Table 5**), suggesting that it is more challenging to utilize hemicellulosic hydrolysates than cellulosic hydrolysates as carbon source for fermentation.

3.7 Implications on cleaner industrial production

Several economic and environmental benefits can be achieved by promoting industrial production of microbial lipids and carotenoids from lignocellulosic biomass. Using cheap and renewable carbon sources to replace edible plants on the production of lipids and carotenoids can avoid the competition with food production. Alternatively, microbial lipids are a promising feedstock to produce biodiesel, which can help to reduce the relying on fossil resources. Moreover, in an industrial scale, the lignin residues from enzymatic hydrolysate can be used as high quality solid biofuels (Larsen et al., 2012), and the yeast cell residues obtained after lipid and carotenoid extraction can serve as nitrogen sources for yeast propagation or anaerobic digestion to produce biogas (Karlsson et al., 2016). Glycerol, the by-product of transesterification of fatty acids to produce biodiesel, can be added in the lipid accumulation stage to increase the carbon to nitrogen ratio (Karlsson et al., 2016). Therefore, the effective utilization of the hemicellulosic and cellulosic fractions of biomass is a key strategy to achieve a “zero waste” and self-sustaining production of lipids and carotenoids.

To advance the industrial production of lignocellulosic biomass derived microbial lipids and carotenoids, more research should be focused on the fermentation and purification steps. Regarding to equipment and facilities, existing bioethanol production equipment, current fermentation facilities and petroleum refinery units can be used for

the production of lipids and carotenoids by oleaginous yeasts without extra investments (Chopra et al., 2019). Improving yeast performance could also be a relevant alternative to enhance the fermentation efficiency. In this sense, increasing the yeast tolerance to inhibitors derived from lignocellulosic biomass can simplify the operation and reduce the investment in the long term. Recently, adaptive laboratory evolution and genetically engineering of yeasts allowed to reach 0.334 g/L h lipid productivity and 0.179 g/g yield by *R. toruloides* cultivated in non-detoxified wheat straw hydrolysate (Díaz et al., 2018). However, different pretreatment methods and different types of biomass may result in different inhibitor's composition and synergistic effects, and it is difficult to build a super strain that could be used in all types of hydrolysates. The same approach can be found for the detoxification method, where there is no standardized and commercialized detoxification method available for different types of hydrolysates. Alternatively, fermentation strategies can also influence the performance of yeasts. For example, high cell density fermentation, fed-batch fermentation, and pre-adaptive fermentation can significantly improve the productivity and tolerance of yeasts (Amza et al., 2019; Fei et al., 2016). Other process steps, such as downstream processing to extract, recover and purify products, should also be studied to efficiently separate lipids and carotenoids. Further research on upstream to downstream are therefore needed to achieve the industrial production.

4. Conclusion

The feasibility of using cellulosic and hemicellulosic hydrolysates to produce lipids and carotenoids by oleaginous yeasts was demonstrated in this work. Besides being a more sustainable option for the production of these compounds, this is also

important because the substitution of expensive substrates by lignocellulosic hydrolysates may lead to a reduction in the production costs at industrial scale. *L. starkeyi* and *R. toruloides* were identified as strains with great ability to produce lipids and carotenoids, respectively, from lignocellulosic hydrolysates. However, an efficient utilization of both cellulosic and hemicellulosic hydrolysates was achieved when they were submitted to detoxification with activated charcoal, revealing that inhibitors have an important influence in the bioconversion process. It was demonstrated that lowering inhibitory levels improves the sustainable processing of biomass for lipids and carotenoids production. In this sense, the choice of the detoxification method is a relevant aspect to take into account in order to avoid extra costs for the overall process. Activated charcoal, as proposed here, is a low cost method that could be easily implemented on an industrial scale. The development of stronger strains by adaptive laboratory evolution and/or genetic modification could also be another possible way to overcome the inhibition caused by toxic compounds as well as their synergistic effects when in presence of high sugar concentration, without offering extra costs for the overall process.

Acknowledgements

This work was supported by the Novo Nordisk Foundation (NNF), Denmark (grant number NNF10CC1016517); the China Scholarship Council (CSC); and the Agencia Nacional de Investigación e Innovación (ANII), Uruguay (project number FSE_S_2017_1_144465).

References

- Alleman, T.L., McCormick, R.L., Christensen, E.D., Fioroni, G., Moriarty, K., Yanowitz, J., 2016. Biodiesel Handling and Use Guide, Related Information: Supercedes September 2008 version. <https://doi.org/10.2172/938562>
- Amza, R.L., Kahar, P., Juanssilfero, A.B., Miyamoto, N., Otsuka, H., Kihira, C., Ogino, C., Kondo, A., 2019. High cell density cultivation of *Lipomyces starkeyi* for achieving highly efficient lipid production from sugar under low C/N ratio. *Biochem. Eng. J.* 149, 107236. <https://doi.org/10.1016/j.bej.2019.05.013>
- Ansari, R., Mohammad-Khah, A., 2009. Activated Charcoal : Preparation , characterization and Applications : A review article. *Int. J. ChemTech Res.* 1, 859–864. <https://doi.org/ISSN 0974-4290>
- Arous, F., Frikha, F., Triantaphyllidou, I.E., Aggelis, G., Nasri, M., Mechichi, T., 2016. Potential utilization of agro-industrial wastewaters for lipid production by the oleaginous yeast *Debaryomyces etchellsii*. *J. Clean. Prod.* 133, 899–909. <https://doi.org/10.1016/j.jclepro.2016.06.040>
- Ballesteros, L.F., Teixeira, J.A., Mussatto, S.I., 2014. Selection of the Solvent and Extraction Conditions for Maximum Recovery of Antioxidant Phenolic Compounds from Coffee Silverskin. *Food Bioprocess Technol.* 7, 1322–1332. <https://doi.org/10.1007/s11947-013-1115-7>
- Beopoulos, A., Nicaud, J.M., Gaillardin, C., 2011. An overview of lipid metabolism in yeasts and its impact on biotechnological processes. *Appl. Microbiol. Biotechnol.* 90, 1193–1206. <https://doi.org/10.1007/s00253-011-3212-8>
- Brandenburg, J., Blomqvist, J., Pickova, J., Bonturi, N., Sandgren, M., Passoth, V., 2016. Lipid production from hemicellulose with *Lipomyces starkeyi* in a pH

- regulated fed-batch cultivation. *Yeast* 33, 451–462. <https://doi.org/10.1002/yea.3160>
- Chen, X., Li, Z., Zhang, X., Hu, F., Ryu, D.D.Y., Bao, J., 2009. Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. *Appl. Biochem. Biotechnol.* 159, 591–604. <https://doi.org/10.1007/s12010-008-8491-x>
- Díaz, T., Fillet, S., Campoy, S., Vázquez, R., Viña, J., Murillo, J., Adrio, J.L., 2018. Combining evolutionary and metabolic engineering in *Rhodospiridium toruloides* for lipid production with non-detoxified wheat straw hydrolysates. *Appl. Microbiol. Biotechnol.* 102, 3287–3300. <https://doi.org/10.1007/s00253-018-8810-2>
- Fan, J., Santomauro, F., Budarin, V.L., Whiffin, F., Abeln, F., Chantasuban, T., Gore-Lloyd, D., Henk, D., Scott, R.J., Clark, J., Chuck, C.J., 2018. The additive free microwave hydrolysis of lignocellulosic biomass for fermentation to high value products. *J. Clean. Prod.* 198, 776–784. <https://doi.org/10.1016/j.jclepro.2018.07.088>
- Fei, Q., O'Brien, M., Nelson, R., Chen, X., Lowell, A., Dowe, N., 2016. Enhanced lipid production by *Rhodospiridium toruloides* using different fed-batch feeding strategies with lignocellulosic hydrolysate as the sole carbon source. *Biotechnol. Biofuels* 9, 1–12. <https://doi.org/10.1186/s13068-016-0542-x>
- Frengova, G.I., Beshkova, D.M., 2009. Carotenoids from *Rhodotorula* and *Phaffia*: Yeasts of biotechnological importance. *J. Ind. Microbiol. Biotechnol.* 36, 163–180. <https://doi.org/10.1007/s10295-008-0492-9>
- Gupta, R., Mehta, G., Kuhad, R.C., 2016. Scale-up of abatement of fermentation inhibitors from acid hydrolysates for efficient conversion to ethanol as biofuel. *J.*

- Chem. Technol. Biotechnol. 91, 1826–1834. <https://doi.org/10.1002/jctb.4775>
- Hu, C., Zhao, X., Zhao, J., Wu, S., Zhao, Z.K., 2009. Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodospiridium toruloides*. *Bioresour. Technol.* 100, 4843–4847. <https://doi.org/10.1016/j.biortech.2009.04.041>
- Izard, J., Limberger, R.J., 2003. Rapid screening method for quantitation of bacterial cell lipids from whole cells. *J. Microbiol. Methods* 55, 411–418. [https://doi.org/10.1016/S0167-7012\(03\)00193-3](https://doi.org/10.1016/S0167-7012(03)00193-3)
- Jule Brandenburg, Johanna Blomqvist, Jana Pickova, Nemailla Bonturi, Mats Sandgren, olkmar P., 2016. Lipid production from hemicellulose with *Lipomyces starkeyi* in a pH regulated fed-batch cultivation. *Yeast* 33, 451–462. <https://doi.org/10.1002/yea.3160>
- Karlsson, H., Ahlgren, S., Sandgren, M., Passoth, V., Wallberg, O., Hansson, P.A., 2016. A systems analysis of biodiesel production from wheat straw using oleaginous yeast: process design, mass and energy balances. *Biotechnol. Biofuels* 9, 1–13. <https://doi.org/10.1186/s13068-016-0640-9>
- Larsen, J., Haven, M.Ø., Thirup, L., 2012. Inbicon makes lignocellulosic ethanol a commercial reality. *Biomass and Bioenergy* 46, 36–45. <https://doi.org/10.1016/j.biombioe.2012.03.033>
- Mata-Gómez, L.C., Montañez, J.C., Méndez-Zavala, A., Aguilar, C.N., 2014. Biotechnological production of carotenoids by yeasts: An overview. *Microb. Cell Fact.* 13, 1–11. <https://doi.org/10.1186/1475-2859-13-12>
- Mussatto, S.I., Dragone, G., Roberto, I.C., 2005. Influence of the toxic compounds present in brewer's spent grain hemicellulosic hydrolysate on xylose-to-xylitol bioconversion by *Candida guilliermondii*. *Process Biochem.* 40, 3801–3806.

- <https://doi.org/10.1016/j.procbio.2005.06.024>
- Mussatto, S.I., Fernandes, M., Rocha, G.J.M., Órfão, J.J.M., Teixeira, J.A., Roberto, I.C., 2010. Production, characterization and application of activated carbon from Brewer's spent grain lignin. *Bioresour. Technol.* 101, 2450–2457.
- <https://doi.org/10.1016/j.biortech.2009.11.025>
- Mussatto, S.I., Roberto, I.C., 2004. Kinetic behavior of *Candida guilliermondii* yeast during xylitol production from highly concentrated hydrolysate. *Process Biochem.* 39, 1433–1439. [https://doi.org/10.1016/S0032-9592\(03\)00261-9](https://doi.org/10.1016/S0032-9592(03)00261-9)
- Ounine, K; Petitedemange, H; Raval, G; Gay, R., 1985. Regulation and Butanol Inhibition of D-Xylose. *Microbiology* 49, 874–878.
- Patel, A., Arora, N., Sartaj, K., Pruthi, V., Pruthi, P.A., 2016. Sustainable biodiesel production from oleaginous yeasts utilizing hydrolysates of various non-edible lignocellulosic biomasses. *Renew. Sustain. Energy Rev.* 62, 836–855.
- <https://doi.org/10.1016/j.rser.2016.05.014>
- Probst, K. V., Schulte, L.R., Durrett, T.P., Rezac, M.E., Vadhani, P. V., 2016. Oleaginous yeast: a value-added platform for renewable oils. *Crit. Rev. Biotechnol.* 36, 942–955. <https://doi.org/10.3109/07388551.2015.1064855>
- Qadeer, S., Khalid, A., Mahmood, S., Anjum, M., Ahmad, Z., 2017. Utilizing oleaginous bacteria and fungi for cleaner energy production. *J. Clean. Prod.* 168, 917–928. <https://doi.org/10.1016/j.jclepro.2017.09.093>
- Qin, L., Li, W.C., Liu, L., Zhu, J.Q., Li, X., Li, B.Z., Yuan, Y.J., 2016. Inhibition of lignin-derived phenolic compounds to cellulase. *Biotechnol. Biofuels* 9, 1–10.
- <https://doi.org/10.1186/s13068-016-0485-2>
- Rodriguez-amaya, D.B., 2001. A guide to carotenoid analysis in foods. ILSI press,

Washington.

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., Sousa, L. da C., Dale, B.E., 2016.

Comparative lipid production by oleaginous yeasts in hydrolyzates of

lignocellulosic biomass and process strategy for high titers. *Biotechnol. Bioeng.*

113, 1676–1690. <https://doi.org/10.1002/bit.25928>

Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre,

S.B., Keasling, J.D., 2010. Microbial production of fatty-acid-derived fuels and

chemicals from plant biomass. *Nature* 463, 559–562.

<https://doi.org/10.1038/nature08721>

The American Oil Chemists' Society, 1998a. Preparation of methyl esters or fatty acids

Ce 2-66, in: AOCS Official Method.

The American Oil Chemists' Society, 1998b. Fatty Acid Composition by GLC Ce 1b-

89, in: AOCS Official Method. AOCS Champaign.

Vasconcelos, B., Teixeira, J.C., Dragone, G., Teixeira, J.A., 2019. Oleaginous yeasts for

sustainable lipid production—from biodiesel to surf boards, a wide range of

“green” applications. *Appl. Microbiol. Biotechnol.* [https://doi.org/10.1007/s00253-](https://doi.org/10.1007/s00253-019-09742-x)

019-09742-x

Wang, S., Sun, X., Yuan, Q., 2018. Strategies for enhancing microbial tolerance to

inhibitors for biofuel production: A review. *Bioresour. Technol.* 258, 302–309.

<https://doi.org/10.1016/j.biortech.2018.03.064>

Yu, X., Zheng, Y., Dorgan, K.M., Chen, S., 2011. Oil production by oleaginous yeasts

using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid.

Bioresour. Technol. 102, 6134–6140.

<https://doi.org/10.1016/j.biortech.2011.02.081>

Zhao, X., Kong, X., Hua, Y., Feng, B., Zhao, Z., 2008. Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. *Eur. J. Lipid Sci. Technol.* 110, 405–412.

<https://doi.org/10.1002/ejlt.200700224>

Zhao, X., Peng, F., Du, W., Liu, C., Liu, D., 2012. Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *Rhodospiridium toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid. *Bioprocess Biosyst. Eng.* 35, 993–1004. <https://doi.org/10.1007/s00449-012-0684-6>

Figure Captions

Fig. 1. Schematic representation of the preparation of cellulosic and hemicellulosic hydrolysates from wheat straw.

Fig. 2. Fermentation of decolorized cellulosic hydrolysate (DCH) by (A) *R. toruloides* and (B) *L. starkeyi* at 30 °C and 250 rpm.

Fig. 3. Fermentation of washed cellulosic hydrolysate (WCH) by (A) *R. toruloides* and (B) *L. starkeyi* at 30 °C and 250 rpm.

Fig. 4. Fermentation of concentrated and decolorized cellulosic hydrolysate (CDCH) by *R. toruloides* (A) and synthetic concentrated medium (SCM) by *R. toruloides* (B) and *L. starkeyi* (C), at 30 °C and 250 rpm.

Fig. 5. Fermentation of post hydrolyzed, 2-fold concentrated and decolorized hemicellulosic hydrolysate (C2DH) by (A) *R. toruloides* and (B) *L. starkeyi* at 30 °C and 250 rpm.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

--

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Highlights

- *L. starkeyi* and *R. toruloides* were the best producers of lipids and carotenoids
- Performance of *L. starkeyi* was not significantly influenced by C:N ratios 25 to 100
- *R. toruloides* showed better fermentation performance under higher C:N ratios
- Yeasts utilized cellulosic and hemicellulosic hydrolysates without nutrient addition
- Phenolics and high sugars concentration (>115 g/L) affected the yeasts' performance

Table 1. Cell mass concentration, lipid and carotenoid production by different oleaginous yeasts cultivated in synthetic media at 30 °C, 250 rpm for 120 h.

Yeast	C:N ratio	Cell mass concentration (g/L)	Lipid concentration (g/L)	Lipid accumulation (%)	Carotenoid concentration (mg/L)	Carotenoid accumulation (mg/g)
Glucose						
<i>R. glutinis</i>	25	1.23±0.23	0.10±0.04	9.55±5.22	2.96±0.16	2.44±0.35
	50	1.28±0.04	0.17±0.07	8.12±4.00	1.81±0.07	2.85±0.08
	100	1.02±0.19	0.10±0.04	8.36±2.68	2.62±0.20	2.573±0.21
<i>R. toruloides</i>	25	3.90±0.23	0.42±0.08	6.16±2.29	7.61±1.61	1.95±0.34
	50	5.31±0.64	0.38±0.04	6.61±1.80	0.94±0.22	2.06±0.04
	100	5.98±0.38	1.20±0.16	20.81±1.85	6.65±1.01	2.23±0.37
<i>S. coloradoensis</i>	25	5.52±0.98	0.03±0.01	6.43±0.23	6.66±0.70	1.24±0.28
	50	8.30±2.26	0.69±0.19	9.33±1.68	6.02±0.35	0.72±0.04
	100	3.37±0.20	0.26±0.06	6.49±3.06	2.89±0.53	1.72±0.35
<i>L. starkeyi</i>	25	11.73±0.18	3.11±0.24	26.54±1.77		
	50	10.07±0.95	3.03±0.24	30.10±1.61		
	100	11.12±0.33	3.24±0.25	29.14±0.17		
<i>Y. lipolytica</i> (YB-392)	25	3.50±0.13	0.23±0.05	7.44±3.00		
	50	5.47±2.11	0.61±0.09	10.05±2.71		
	100	6.22±0.28	2.46±0.25	39.52±1.36		
<i>Y. lipolytica</i> (YB-567)	25	3.55±0.18	0.22±0.03	8.85±2.82		
	50	4.73±0.25	0.57±0.12	12.00±0.88		
	100	6.18±0.16	2.25±0.21	36.39±2.08		
Xylose						
<i>R. toruloides</i>	25	2.85±0.53	0.14±0.02	2.63±0.51	8.30±0.49	2.99±0.66
	50	3.42±0.20	0.26±0.07	4.12±1.01	9.23±0.97	2.70±0.28
	100	6.27±0.14	2.20±0.25	35.01±3.54	6.21±0.07	2.88±0.15
<i>S. coloradoensis</i>	25	5.83±0.45	0.45±0.16	8.58±2.07	5.81±0.43	0.90±0.17
	50	6.12±0.85	0.69±0.11	12.98±0.58	6.55±0.41	1.07±0.07
	100	5.88±0.60	0.53±0.07	9.60±0.47	4.38±0.54	1.49±0.20
<i>L. starkeyi</i>	25	12.17±0.08	3.60±0.27	29.62±1.40		
	50	11.88±1.38	3.61±0.40	30.38±3.87		
	100	11.78±0.08	3.49±0.46	29.59±2.51		

Table 2. Main fatty acids produced by the different oleaginous yeasts cultivated in synthetic media at 30 °C, 250 rpm for 120 h. Values correspond to % of the total fatty acids produced.

C:N ratio	Fatty acid	<i>R. glutinis</i>	<i>R. toruloides</i>	<i>S. coloradoensis</i>	<i>L. starkeyi</i>	<i>Y. lipolytica</i> (YB-392)	<i>Y. lipolytica</i> (YB-567)
25	Glucose						
	C16:0	18.44±0.10	17.81±0.15	16.94± 0.35	30.44±0.16	15.99±1.04	16.69±1.08
	C18:0	7.10±0.05	9.56±0.05	6.64±0.32	7.94±0.23	7.64±0.35	7.73±0.33
	C18:1(n-9)	20.29±0.40	14.17±0.08	17.00±0.24	37.10±1.63	32.88±0.76	30.17±1.44
50	C18:2(n-6)	17.12±0.21	12.91±0.05	28.94±0.69	5.34±0.13	10.63±0.51	10.42±0.29
	C16:0	19.89±0.32	17.07±1.59	19.71±0.46	31.44±0.88	17.72±0.01	17.22±0.47
	C18:0	7.94±0.09	10.39±0.95	8.56±0.10	8.48±0.24	10.95±0.03	9.40±0.14
	C18:1(n-9)	20.63±0.24	15.84±1.44	10.38±1.01	31.29±0.47	22.5±0.02	25.58±1.02
100	C18:2(n-6)	15.35±0.01	10.57±0.94	21.47±0.88	5.82±0.13	8.77±0.05	10.57±0.27
	C16:0	17.78±0.47	22.8±0.25	20.31±1.03	30.40±0.41	19.51±0.61	16.93±0.34
	C18:0	7.53±0.36	20.66±3.22	9.75±0.60	11.17±0.60	11.00±0.33	7.57±0.11
	C18:1(n-9)	25.64±1.19	31.86±3.73	8.88±0.22	33.96±1.41	28.39±0.96	29.65±0.50
25	Xylose						
	C18:2(n-6)	16.19±0.61	15.25±0.23	13.67±0.34	4.12±0.02	12.38±0.41	13.33±0.25
	C16:0		16.94±0.35	20.15±1.57	29.18±1.05		
	C18:0		7.54±2.47	5.29±0.76	9.71±0.26		
50	C18:1(n-9)		14.51±0.96	20.63±0.38	34.43±1.02		
	C18:2(n-6)		18.70±9.86	34.06±2.12	4.94±0.15		
	C16:0		17.94±1.72	17.39±0.46	29.72±1.15		
	C18:0		10.88±1.12	5.45±0.10	10.21±0.16		
100	C18:1(n-9)		18.90±2.03	13.24±1.01	34.42±1.69		
	C18:2(n-6)		10.08±1.03	40.57±0.88	4.33±0.28		
	C16:0		23.89±0.19	19.30±1.37	24.24±0.31		
	C18:0		16.60±2.99	7.36±0.35	19.93±0.14		
	C18:1(n-9)		35.86±3.43	11.16±0.51	26.76±0.41		
	C18:2(n-6)		13.97±0.16	31.28±1.89	2.80±0.00		

Table 3. Composition of different cellulosic hydrolysates used for cultivation of *L. starkeyi* and *R. toruloides*.

Compound	Concentration (g/L)					
	CH	DCH	CCH	CDCH	WCH	WCCH
Glucose	43.96±0.09	43.60±0.01	95.52±0.67	89.09±2.04	46.40±0.18	111.97±0.67
Xylose	12.51±0.03	12.34±0.03	28.64±0.51	26.36±0.16	11.36±0.02	23.88±0.82
Acetic acid	1.19±0.03	1.14±0.03	2.15±0.04	2.00±0.21	0.63±0.03	1.12±0.03
HMF	-	-	0.02±0.00	-	-	0.02±0.00
Furfural	0.19±0.01	0.01±0.00	0.02±0.00	-	-	0.01±0.00
Phenolic compounds	1.45±0.07	0.19±0.02	2.35±0.16	0.57±0.03	0.42±0.04	0.96±0.03

CH: Raw cellulosic hydrolysate; DCH: Decolorized cellulosic hydrolysate; CCH: Concentrated cellulosic hydrolysate; CDCH: Concentrated and decolorized cellulosic hydrolysate; WCH: Washed cellulosic hydrolysate; WCCH: Washed and concentrated cellulosic hydrolysate

- Undetected

Table 4. Composition of different hemicellulosic hydrolysates used for cultivation of *L. starkeyi* and *R. toruloides*.

Compound	Concentration (g/L)					
	HH	PH	C2H	C2DH	C4H	C4DH
Glucose	0.08±0.03	2.91±0.45	4.72±0.58	4.10±0.63	13.15±0.77	11.53±0.20
Xylose	0.99±0.01	14.20±0.04	24.76±0.16	23.89±0.15	54.21±1.91	51.63±0.20
Acetic acid	1.40±0.01	2.05±0.08	3.67±0.05	3.57±0.15	6.30±0.04	4.86±0.11
HMF	-	0.01±0.00	-	-	0.34±0.01	0.02±0.00
Furfural	0.61±0.10	0.70±0.09	0.07±0.01	-	-	-
Phenolic	1.37±0.04	1.60±0.02	2.52±0.06	0.63±0.14	4.29±0.04	0.83±0.03

HH: Raw hemicellulosic hydrolysate; PH: Post hydrolyzed hemicellulosic hydrolysate; C2H: Post hydrolyzed and 2-fold concentrated hemicellulosic hydrolysate; C2DH:

Post hydrolyzed, 2-fold concentrated and decolorized hemicellulosic hydrolysate; C4H: Post hydrolyzed and 4-fold concentrated hemicellulosic hydrolysate; C4DH:

Post hydrolyzed, 4-fold concentrated and decolorized hemicellulosic hydrolysate

- Undetected

Table 5. Comparison of fermentation parameters for *R. toruloides* and *L. starkeyi* cultivated in synthetic media and cellulosic / hemicellulosic hydrolysate

Media	Cell mass	Lipid				Carotenoid			
	Concentration (g/L)	Concentration (g/L)	Accumulation (%)	Yield (g/g)	Productivity (mg/L h)	Concentration (mg/L)	Accumulation (mg/g)	Yield (mg/g)	Productivity (mg/L h)
<i>R. toruloides</i>									
DCH	17.95±0.42	3.36±0.29	18.73±1.02	0.05±0.01	34.98±3.00	24.58±1.88	1.37±0.07	0.32±0.03	0.26±0.02
WCH	8.37±0.23	2.09±0.30	24.95±3.01	0.06±0.01	17.42±2.52	21.42±2.04	2.57±0.32	0.62±0.06	0.18±0.02
CDCH	6.60±0.67	0.61±0.01	9.31±0.09	0.02±0.00	5.12±0.05	3.48±0.44	0.53±0.07	0.14±0.02	0.03±0.00
C2DH	10.73±0.56	1.01±0.02	9.44±0.42	0.03±0.00	8.43±0.20	15.80±0.56	1.48±0.52	0.52±0.02	0.13±0.00
SCM	4.50±0.40	0.36±0.04	8.09±0.41	0.01±0.00	3.03±0.30	8.14±1.16	1.82±0.34	0.24±0.03	0.07±0.01
<i>L. starkeyi</i>									
DCH	12.57±0.20	3.23±0.22	25.67±1.85	0.05±0.04	33.59±2.25				
WCH	17.32±0.12	3.99±0.35	23.02±1.93	0.07±0.01	33.22±2.93				
C2DH	14.10±0.65	1.46±0.99	10.64±0.51	0.05±0.00	12.19±0.77				
SCM	16.75±0.28	3.51±0.37	20.95±1.91	0.05±0.01	29.27±3.11				

DCH: Decolorized cellulosic hydrolysate; WCH: Washed cellulosic hydrolysate; CDCH: Concentrated and decolorized cellulosic hydrolysate; C2DH: Post hydrolyzed, 2-fold concentrated and decolorized hemicellulose hydrolysate; SCM: Synthetic concentrated medium.