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Maximizing the simultaneous production of lipids and carotenoids by *Rhodosporidium toruloides* from wheat straw hydrolysate and perspectives for large-scale implementation

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**Highlights**

- Temperature and inoculum load affected the yeast performance during fermentation.
- Lipid and carotenoid production were maximized under the same fermentation conditions.
- A global production process using wheat straw as a feedstock was proposed.
- Production of lipids and carotenoids by *R. toruloides* showed potential for scaling up.

**Abstract**

This study aimed to select fermentation conditions able to simultaneously maximize the production of lipids and carotenoids by oleaginous yeast cultivated in wheat straw hydrolysate. An evolved strain of *Rhodosporidium toruloides* with improved tolerance to toxic compounds present in hydrolysate medium was used. Experiments were performed in order to investigate the effect of the temperature and inoculum load on the production of lipids and carotenoids by *R. toruloides*. Results revealed that the accumulation of both products can be simultaneously maximized when performing the fermentation at 17 °C and using 3.5 g/L of inoculum. This maximum simultaneous production opens up new perspectives for the establishment of a feasible and more sustainable large-scale process for the production of lipids and carotenoids. Even corresponding to only 1% of the cell mass, due to the high market value, carotenoids would account for more than 90% of the total income of the industrial plant.

**1. Introduction**

Prominent negative consequences caused by the excessive use of fossil resources, including global warming and environmental pollution, represent nowadays big challenges for the humanity. In addition, the increased demand for energy, food and clean water due to the population growth are also aspects contributing to this concern. Finding greener and more sustainable solutions for the industrial processes currently used on a large scale, reducing the emissions of CO\(_2\) and promoting a better utilization of the natural resources and wastes are considered urgent needs today to move towards a cleaner and more sustainable future.

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Single cell oil produced by oleaginous yeasts has been considered as a promising alternative feedstock for the production of biodiesel and oleochemicals. Oleaginous yeasts can accumulate up to 70% of their dry cell weight in lipids, which make them very attractive for industrial application. In addition, lipids accumulated by these yeasts have properties similar to those of vegetable oil, and their production by yeasts do not cause competition with food production (like the vegetable oils, for example) nor depends on the seasons or regions (Liu et al., 2020; Vasconcelos et al., 2019). Carotenoids are also compounds of great interest since they have high economic value and their market is in expansion, being estimated to reach $2.00 billion by 2026 (MarketsandMarkets, 2020). Moreover, carotenoids have numerous applications in food, feed, cosmetic and pharmacy industries, due to their relevant properties such as anti-oxidant and anti-tumor activities, and attractive color (Novoveska et al., 2019).

To promote the industrialization of microbial lipid and carotenoid production, efforts are required in different aspects of the production chain, including the selection and/or development of a microbial strain with potential to be used in large-scale processes, the establishment of an appropriate bioprocess able to maximize the product formation by the yeast, and the development of an efficient downstream processing to separate lipids and carotenoids. In addition, the utilization of cheap and renewable carbon sources for the yeast cultivation is a key point to be considered in order to have a more sustainable technology (Yamakawa et al., 2020). Lignocellulosic biomass is rich in glucose and xylose sugars, which can be used as carbon source for fermentation (Musatso and Dragone, 2016). Moreover, lignocellulosic biomass can be obtained from agricultural residues, forest and energy crops. Among them, wheat straw has been considered as an important feedstock for fermentation purposes, as it is widely available throughout the world. In Europe, for example, the average annual production of wheat straw is 109 million tons, which account for 42.2% of the total crop residues generated in this area (Scarlat et al., 2010). In turn, oleaginous yeasts are able to utilize different types of carbon source, including glucose, xylose, glycerol and acetic acid (Vasconcelos et al., 2019), being potential candidates for utilization on the conversion of sugars from lignocellulosic biomass. However, due to the compact structure and polymerization of sugars, yeasts are unable to utilize lignocellulosic biomass directly and a pretreatment step is required. Different types of pretreatment have been proposed to overcome the recalcitrance of lignocellulosic biomass (Musatso, 2016), among of which, hydrothermal pretreatment is an environmentally friendly method and was therefore adopted to pretreat wheat straw in the present study.

In our previous studies, Rhodosporidium toruloides was found as being a yeast strain with great ability to convert glucose and xylose from wheat straw hydrolysate into lipids and carotenoids (Liu et al., 2020). Recently, we improved the performance of this yeast to grow and produce lipids and carotenoids from lignocellulosic hydrolysates by applying an adaptive laboratory evolution strategy (Liu et al., 2021a). Additionally, an efficient downstream process to separate lipids and carotenoids was also proposed (Liu et al., 2021b). The present study aims to advance the research in this area by selecting appropriate conditions of fermentation able to result in a maximum simultaneous production of lipids and carotenoids by R. toruloides from wheat straw hydrolysate. This is an important step for future implementation of this microbial process since obtaining a high product formation may help reduce the overall production costs, besides impacting in the downstream processing. In this study, attention was given to define the most suitable conditions of temperature and inoculum load able to maximize the formation of lipids and carotenoids. The experiments were performed using the evolved strain of R. toruloides recently developed in our laboratory, which has improved tolerance to toxic compounds present in wheat straw hydrolysate (Liu et al., 2021a). At the end, the conditions able to maximize the simultaneous production of lipids and carotenoids were selected and a mass balance and a preliminary economic assessment was performed to evaluate the perspectives for large-scale implementation of this bioprocess.

2. Materials and methods

2.1. Wheat straw hydrolysate

Wheat straw used in this study was provided by the Danish Technological Institute (Denmark). The material was supplied in the dried form and had a particle size of 180 to 1800 μm. To prepare the hydrolysate to be used as fermentation medium, wheat straw was initially submitted to a hydrothermal pretreatment (190 °C, 45 min, solid to liquid ratio of 1:10 w/v), which was performed in 600-mL non-stirred pressure vessels (Parr Series 4760, Parr Instrument Company, Moline, IL) heated in a silicone oil bath (Lauda Eco Gold, Germany) as previously described (Liu et al., 2020). After pretreatment, the solid and liquid fractions were separated through a sieve and the solid fraction was hydrolyzed with the cellulase concentrate Cellic C-Tec2, (Novozymes, Bagsværd, Denmark). Hydrolysis experiments were carried out in 1-L Duran laboratory bottles with vertical baffles, which were horizontally accommodated in a Bottle/Tube Roller system (Thermo Scientific, USA) placed inside an incubator, and kept at 50 °C and 50 rpm for 72 h. The conditions used for enzymatic hydrolysis consisted in 10% (w/v) solid load, 15 FPU of enzyme/g dry wheat straw, pH 5.8. Afterwards, the hydrolysate was collected by centrifugation (10000 rpm, 4 °C, 20 min) and stored at 4 °C. To be used as fermentation medium, the hydrolysate was supplemented with 0.25 g/L (NH_{2}SO_{4}, pH adjusted to 4.9 by the addition of 1 M NaOH solution, and sterilized through 0.22 μm membrane.

2.2. Microorganism and pre-cultivation conditions

An evolved strain of the yeast Rhodosporidium toruloides with improved ability to grow in wheat straw hydrolysate, previously obtained via adaptive laboratory evolution in our laboratory (Liu et al., 2021a), was used in the experiments. The strain was stored at –80 °C in 20% glycerol.

In the pre-cultivation (reactivation) step, one loop of yeast cells was transferred to 100 mL of a medium composed of 50 g/L glucose, 1.7 g/L yeast nitrogen base without amino acid and ammonium sulfate, and 1 g/L (NH_{2})_{2}SO_{4} (Sigma-Aldrich), pH 4.9, in 250-mL Erlenmeyer flasks and cultivated at 30 °C, 250 rpm for 72 h. Then, to get single colonies, the cell cultures were diluted 1 × 10^{4} folds with sterilized distilled water and 100 μL of the diluted culture was spread on agar plates (prepared with the same medium described above), and incubated at 30 °C for 5 days. Afterwards, the agar plates were maintained at 4 °C in refrigerator and new plates were prepared every two weeks.

2.3. Fermentation conditions

Fermentations were performed in 24-deep well plates containing 3 mL working volume per well. The conditions of inoculum load and temperature used in each fermentation run varied between 0.5 and 3.5 g/L, and between 8 and 22 °C, respectively, and were combined through a 2^{2} central composite design. The ranges of temperature and inoculum load investigated in the present study were set based on preliminary experiments performed in our laboratory with different oleaginous yeasts.

During the experiments, samples were withdrawn every 24 h to measure the absorbance at 600 nm and the °Brix (total soluble solids in an aqueous solution). The experiments were finished when the difference in °Brix for samples taken in two consecutive days was equal or<10%. At the end of each fermentation, the dry cell weight, lipids and carotenoids produced were quantified.
2.4. Analyses

2.4.1. Characterization of wheat straw hydrolysate

The concentrations of glucose, xylene, acetic acid, furfural and 5-hydroxymethylfurfural (5-HMF) in wheat straw hydrolysate 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). The column was eluted with 0.005 M H\textsubscript{2}SO\textsubscript{4}, at 60 °C, with a flow rate of 0.6 mL/min, running for 50 min. Sugars and acetic acid were detected using a refractive index detector, while furfural and 5-HMF were identified using a UV light detector at 280 nm. Phenolic compounds were determined by colorimetric method (Ballesteros et al., 2014) using gallic acid as standard. A Primary nitrogen assay kit and a Urea and ammonia assay kit (Megazyme International, Wicklow, Ireland) were used to measure the nitrogen content in wheat straw hydrolysate.

2.4.2. Dry cell weight measurement, lipid and carotenoid quantification

Dry cell weight quantification was conducted in triplicate for each fermentation condition. For analysis, media samples were centrifuged at 10000 rpm for 5 min. Then, the cell pellets were washed twice with distilled water and dried in an oven at 60 °C until constant weight (approx. 24 h).

Lipid concentration was determined from the wet cell pellets obtained after washing with distilled water, using a sulfo-phospho-vanillin colorimetric assay (Izard and Limberger, 2003). Absorbance readings were performed in a 96-well microplate using a microplate reader (BioTek Synergy Mx, US) at 530 nm. A calibration curve ranging between 0.2 and 1 mg oil/mL was set using sunflower seeds oil (Sigma-Aldrich) dissolved in chloroform as standard.

For carotenoid quantification, cell pellets from 2 mL fermentation samples were collected by centrifugation (5000 rpm, 5 min), washed with distilled water, then suspended in 1 mL Milli-Q water and transferred to bead beating tubes (6914–500, MP Biomedicals, France). Cell disruption was performed on a bead beater (Precells 24, Bertin Technologies, France) for 5 cycles of 60 s at 2500 rpm, with 60 s break after each cycle. After bead beating, all beads, cell debris and liquid in the heating tubes were transferred into glass tubes with screw caps. Then, 2 mL of chloroform were added to each tube containing the disrupted cells and the mixture was sonicated at 80 kHz for 10 min. Afterwards, the tubes were centrifuged at 3500 rpm for 5 min and the bottom phase was taken to another clean glass tube. The extraction was repeated 3 times and the chloroform fractions were combined and dried under nitrogen gas stream. The extracted carotenoids were redissolved in 2 mL acetone containing 0.2% BHT (butylated hydroxytoluene). To find the maximum absorbance wavelength of carotenoids, the absorbance of extracts in acetone was read in a nanophotometer (Implen GmbH, Germany) under a wavelength range between 300 and 900 nm. The maximum absorbance wavelength for carotenoids produced by the evolved R. toruloides yeast was found at 498 nm.

Accumulation of lipids and carotenoids in yeast cells was defined as the ratio of lipids or carotenoids concentration to cell concentration and it was expressed as % for lipids and Abs/g for carotenoids.

2.5. Statistical analysis

A 2\textsuperscript{2} central composite design was used to evaluate the influence of the temperature and inoculum load on the fermentation of wheat straw hydrolysate by R. toruloides. For the experiments, the two independent variables (temperature and inoculum load) were combined in two levels (-1 and +1). Additionally, four experiments were carried out in the center point (0, 0) to estimate the random error needed for the analysis of variance; and other four assays combining the central points with the maximum and minimum levels of the variables were performed to estimate the curvature in the studied region. The volumetric concentration and accumulation of lipids and carotenoids produced were set as responses (dependent variables) of the experimental design. Statistical significance of the variables was determined at 5% probability level ($p < 0.05$). The software Statistica version 10.0 (StatSoft, USA) was used for statistical analysis as well as to obtain the response surfaces and contour plots.

3. Results and discussion

3.1. Composition of wheat straw hydrolysate

The wheat straw hydrolysate used in the present study contained a mixture of hexose (glucose, 29.4 g/L) and pentose sugars (xylene, 13.85 g/L and arabinose, 1.23 g/L), which can be used as carbon source by R. toruloides. Some inhibitory compounds including acetic acid (1.97 g/L), 5-HMF (0.01 g/L), and phenolic compounds (0.79 g/L), were also present in the hydrolysate. These compounds are usually toxic to microorganisms and, depending on the concentration, may have a significant negative impact on the performance of the strain during the fermentation (Musatto and Roberto, 2004). However, the strain of R. toruloides used in the present study was previously evolved to tolerate these inhibitory compounds (Liu et al., 2021a). Therefore, their presence in the medium should not be a problem during the fermentation.

The nitrogen content in the hydrolysate was of 37.06 mg/L (primary amino nitrogen, 20.75 mg/L; urea, 12.28 mg/L; and ammonia, 4.03 mg/L), which resulted in a C:N ratio (concentration base) in the hydrolysate of 1200:1. Previous studies suggested that C:N ratios in the range of 100 to 200 are better for an efficient cell propagation as well as lipid and carotenoid accumulation in oleaginous yeast (Elfeky et al., 2019). Therefore, to be used in the fermentation experiments, the wheat straw hydrolysate was supplemented with 0.25 g/L (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} to reach a C:N ratio of 155.

3.2. Effect of the temperature and inoculum load on cell growth and substrate consumption

The growth profile and substrate consumption (estimated as ‘Bx’, i.e., total soluble solids in the medium) by the yeast during the fermentations using different combinations of temperature and inoculum load, are presented in Fig. 1. According to the results, cell growth and substrate consumption were significantly affected by the temperature and inoculum load used in the experiments. Low temperature and low inoculum load (run 1: 8 °C, 0.5 g/L inoculum) resulted in slower growth rate and substrate consumption compared to the other conditions. A longer lag phase was also observed in this case (Fig. 1A). The results also suggest that the effect of the temperature was more pronounced than the effect of the inoculum since the substrate consumption, for example, was considerably lower for the three runs performed at 8 °C (1, 2 and 3) compared to the runs performed at 22 °C (runs 10, 11, and 12) (Fig. 1B). To better understand the performance of R. toruloides under the different fermentation conditions, the fermentation parameters were calculated (Table 1).

It was noticeable that the highest cell mass (7.45 ± 0.07 g/L) generated by this yeast was obtained at 15 °C, using an inoculum load of 3.5 g/L (run 9, Table 1). This high cell mass concentration resulted in the highest lipid (2.82 ± 0.00 g/L) and carotenoid (0.87 ± 0.05 Abs/L) concentrations among all tested conditions. On the other hand, when the accumulation of lipids and carotenoids was considered, different results were observed. The highest lipid accumulation (43.20 ± 3.16% of dry cell mass) was obtained at 15 °C using 0.5 g/L of inoculum (run 4), while the highest accumulation of carotenoids (0.121 ± 0.004 Abs/g) was obtained when the yeast was cultivated at 8 °C with an inoculum of 3.5 g/L (run 3).
3.3. Selection of the fermentation conditions for maximum simultaneous production of lipids and carotenoids

As lipids and carotenoids are intracellular products of oleaginous yeasts, high cell mass often results in high volumetric concentrations of both compounds. However, a high lipid and carotenoid accumulation in yeast could be relevant for downstream processing, since for the same amount of processed yeasts, more products could be obtained from those cells with higher accumulation abilities, which in turn, could potentially result in less costs related to energy consumption. Therefore, it is important to make a balance between volumetric concentration and accumulation of lipids and carotenoids in the yeast in order to define the most appropriate conditions to be used for fermentation. In this study, both responses, volumetric concentration and accumulation of product per cell were considered of interest for optimization purpose.

When lipid concentration was considered as response of the 2³ central composite design, the highest values were obtained when using the temperature in the range of 16 to 18 °C and inoculum of about 3.5 g/L (Fig. 2A, B). Similarly, temperatures between 16 and 18 °C and 3.5 g/L of inoculum load led to higher lipid accumulation by R. toruloides (Fig. 2C, D). The effect estimation and analysis of variance (ANOVA) confirmed that both variables, temperature and inoculum load, had significant effects (p < 0.05) on lipid concentration. Nevertheless, for lipid accumulation, only the effect of the temperature was significant. Based on statistical analysis, the temperature of 17 °C and inoculum load of 3.5 g/L were selected as optimum for both lipid concentration and lipid accumulation by R. toruloides.

Similar to the lipid concentration, the highest values of carotenoid concentration were also obtained when a temperature of approx. 17 °C and inoculum load of 3.5 g/L (Fig. 2A, B) were used, and, according to the effect estimation and ANOVA analysis, both variables had significant effects (p < 0.05) on carotenoid concentration. On the other hand, the carotenoid accumulation was improved when the temperature was reduced to 8 °C, but still using an inoculum of 3.5 g/L (Fig. 3C, D). In fact, the carotenoid accumulation was significantly affected (p < 0.05) by the inoculum load within the range of values tested (0.5 g/L to 3.5 g/L), while the effect of the temperature was not significant within the range of values studied (8 to 22 °C). Based on the above, 17 °C and 3.5 g/L

| Run | Temperature (°C) | Inoculum (g/L) | Available Nitrogen (g/g cells) | Total cells (g/L) | Produced cells (g/L) | Lipid concentration (g/L) | Lipid accumulation (g/L) | Carotenoid concentration (Abs/mL) | Carotenoid accumulation (Abs/g cell) |
|-----|-----------------|----------------|-------------------------------|-----------------|---------------------|------------------------|------------------------|-------------------------------|----------------------------------|
| 1   | 8               | 0.5            | 0.57                          | 3.35 ± 0.21     | 2.85 ± 0.21         | 5.6                    | 0.79 ± 0.02             | 23.66 ± 0.55                  | 0.30 ± 0.03                     | 0.089 ± 0.009                    |
| 2   | 8               | 2              | 0.14                          | 4.40 ± 0.14     | 2.4 ± 0.14          | 5.5                    | 1.28 ± 0.02             | 28.98 ± 0.36                  | 0.52 ± 0.03                     | 0.119 ± 0.007                    |
| 3   | 8               | 3.5            | 0.08                          | 5.60 ± 0.28     | 2.1 ± 0.28          | 5.2                    | 1.67 ± 0.03             | 29.83 ± 0.56                  | 0.68 ± 0.02                     | 0.121 ± 0.004                    |
| 4   | 15              | 0.5            | 0.57                          | 4.75 ± 0.07     | 4.25 ± 0.07         | 4.1                    | 2.05 ± 0.15             | 43.20 ± 3.16                  | 0.46 ± 0.05                     | 0.098 ± 0.01                     |
| 5   | 15              | 2              | 0.14                          | 6.05 ± 0.07     | 4.05 ± 0.07         | 3.7                    | 2.44 ± 0.00             | 40.30 ± 0.00                  | 0.68 ± 0.02                     | 0.112 ± 0.004                    |
| 6   | 15              | 2              | 0.14                          | 6.30 ± 0.14     | 4.30 ± 0.14         | 3.8                    | 2.41 ± 0.21             | 38.31 ± 3.39                  | 0.69 ± 0.03                     | 0.109 ± 0.005                    |
| 7   | 15              | 2              | 0.14                          | 6.05 ± 0.07     | 4.05 ± 0.07         | 3.7                    | 2.33 ± 0.02             | 38.57 ± 0.35                  | 0.68 ± 0.07                     | 0.112 ± 0.011                    |
| 8   | 15              | 2              | 0.14                          | 6.25 ± 0.21     | 4.25 ± 0.21         | 3.7                    | 2.36 ± 0.04             | 37.75 ± 0.59                  | 0.69 ± 0.03                     | 0.111 ± 0.005                    |
| 9   | 15              | 3.5            | 0.08                          | 7.45 ± 0.07     | 3.95 ± 0.07         | 3.3                    | 2.82 ± 0.00             | 37.83 ± 0.00                  | 0.87 ± 0.05                     | 0.116 ± 0.007                    |
| 10  | 22              | 0.5            | 0.14                          | 4.65 ± 0.07     | 4.15 ± 0.07         | 2.9                    | 1.63 ± 0.06             | 35.08 ± 1.30                  | 0.47 ± 0.05                     | 0.100 ± 0.010                    |
| 11  | 22              | 2              | 0.57                          | 5.55 ± 0.64     | 3.55 ± 0.64         | 2.6                    | 1.97 ± 0.08             | 35.47 ± 1.52                  | 0.59 ± 0.07                     | 0.105 ± 0.013                    |
| 12  | 22              | 3.5            | 0.08                          | 6.10 ± 0.00     | 2.6 ± 0.00          | 2.3                    | 2.48 ± 0.23             | 40.63 ± 3.80                  | 0.71 ± 0.05                     | 0.116 ± 0.008                    |

**Table 1**

Cell mass, Brix, lipid and carotenoid production at the endpoint of fermentations of wheat straw hydrolysate by *R. toruloides* under different conditions of temperature and inoculum load according to a 2³ central composite design.

**Fig. 1.** Growth profile (A) and content of total soluble solids (B) in wheat straw hydrolysate during the fermentation with *R. toruloides*. T: temperature (°C), In: inoculum load (g/L).

3.3. Selection of the fermentation conditions for maximum simultaneous production of lipids and carotenoids
L of inoculum load were considered as the most appropriate conditions of fermentation to obtain maximum simultaneous production of lipids and carotenoids by *R. toruloides* from wheat straw hydrolysate.

It is worth noting that a temperature of 30 °C has been usually considered for the production of lipids and carotenoids by fermentation with oleaginous yeasts (Saran et al., 2017; Soccol et al., 2017). However, the present study revealed that a lower temperature (17 °C) is more suitable to improve the production of lipids and carotenoids by *R. toruloides*. Reducing the temperature from 28 to 20 °C was also recently reported as being beneficial for the accumulation of lipids and carotenoids by *Rhodotorula* yeast (Kot et al., 2019). Nevertheless, it is important to highlight that, in the case of the carotenoids, temperature variations may change the biosynthetic pathway of these compounds in yeast (Frengova and Beshkova, 2009) since β-carotene synthetase and torulene synthetase are temperature-dependent enzymes (Hayman et al., 1974). High temperature (30 °C) is beneficial to the synthesis of torulene and torularhodin in oleaginous yeasts, while more β-carotene can be generated at low temperature (20 °C) (Frengova and Beshkova, 2009; Hayman et al., 1974; Kot et al., 2019).

3.4. Perspectives for large-scale implementation and impact

Based on the results of the present study and our previous studies on this topic (Liu et al., 2021a, 2021b, 2020), a global process for the production of lipids and carotenoids from oleaginous yeast using wheat straw as a feedstock was proposed. Fig. 4 shows a schematic representation of this global process. In the proposed production route, a hydrothermal process, in which only water and no extra chemicals is
added, is used for initial pretreatment of the biomass. Then, the solid fraction of pretreated wheat straw is hydrolyzed with enzymes to produce a cellulosic hydrolysate, which will be used as carbon source for fermentation as obtained, i.e., without detoxification, using the evolved strain of the oleaginous yeast *R. toruloides* (Liu et al., 2021a). The remaining solid after enzymatic hydrolysis is mainly composed by lignin and small amounts of cellulose and hemicellulose, and can be considered as a high quality solid for heat generation (Castro et al., 2019; Larsen et al., 2012). For fermentation, the optimum conditions of temperature and inoculum load, as established in this study, are used to obtain maximum production of lipids and carotenoids simultaneously. Once the fermentation is finished, the cell mass is collected from the fermentation broth, and follows to a downstream processing. A saponification method using whole wet cells is used to extract and separate lipids and carotenoids from the yeast cells (Liu et al., 2021b). After this step, lipids and carotenoids are both recovered with high yield; while the remaining cell debris can be recycled for animal feed or used as nutrient source for fermentation (Louhasakul et al., 2018). The solvent used for lipid and carotenoid extraction can also be recycled for further use, demonstrating that this is an almost zero-waste and self-sustaining purification method.

For the mass balances, considering a microbial biodiesel production plant with an annual productivity of 1,000 t, 80% conversion ratio of lipids into biodiesel (Koutinas et al., 2014) and 86% lipid recovery, at least 1,453 t of lipids need to be produced each year. Considering the evolved strain of *R. toruloides* (Liu et al., 2021a), the lipid and carotenoid accumulation obtained when the strain was cultivated in wheat straw hydrolysate corresponded to 28% of dry cell weight and 1% of dry cell weight (10 mg/g cell), respectively. This means that around 5,189 t of cell mass need to be generated to result in an annual production of lipids equal to 1,453 t. As a result, a carotenoid yield of approx. 52 t would be obtained per year. Considering a carotenoid recovery of 79% (Liu et al., 2021b), 41 t of carotenoids could be obtained in this plant as an extra product. To generate 5,189 t of cells, when the cell mass concentration in the hydrolysate was 16 g/L (Liu et al., 2021b), 324,400 t of hydrolysate is required. The saccharification efficiency was around 74% under this enzymatic hydrolysis condition (Liu et al., 2020), as a result, 8,224 t of lignin residue is obtained, which can be used for heat generation. Based on the pretreatment parameters, around 12% of wheat straw was liquefied during pretreatment, and the pretreated wheat straw contained

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**Fig. 3.** Carotenoid concentration (A, B) and accumulation (C, D) by *R. toruloides* as a function of the temperature and inoculum load used for fermentation. (A, C) response surfaces; (B, D) contour plots.
Fig. 4. Global process proposed for the production of lipids and carotenoids from oleaginous yeast using wheat straw as a feedstock.
76% of moisture (Liu et al., 2020). So, to obtain 324,400 t of hydrolysate, 37,752 t of wheat straw were required.

It is worth noting that lipids produced by evolved R. toruloides cultivated in wheat straw hydrolysate are mainly composed of 42% of palmitic acid (C16:0), 15% of stearic acid (C18:0) and 39% of oleic acid (C18:1), being suitable for use in the production of biodiesel (Liu et al., 2021b). Carotenoids accumulated by this yeast were composed of 4.7% of γ-carotene, 52.4% of ß-carotene and 42.9% of torulene, which have potential to be used in food, feed, pharmaceutical and cosmetic industries (Liu et al., 2021b; Novojeska et al., 2019).

Microbial lipids and carotenoids are still considered expensive products. However, comparison of the above proposed process and results with literature data suggests that the microbial production of lipids and carotenoids by R. toruloides using lignocellulosic biomass could be a feasible and sustainable process able to be scaled. When glucose was used as carbon source to produce microbial biodiesel, for example, the production cost of the biodiesel was estimated in $5,900/t (Koutinas et al., 2014). On the other hand, the production cost of the biodiesel derived from vegetable oil was calculated in $1,437.5/t without considering the glycerol credit (Ong et al., 2012). Regarding the microbial production of carotenoids, for the best scenario, the cost for microalgae derived astaxanthin was estimated in $1,830/kg, which was uncompetitive when compared to the artificial synthetic one ($1,049/kg) (Panis and Carreon, 2016). Recovering both lipids and carotenoids during the same production process, as proposed in the present study, may change this situation.

To compare the economic performance of biodiesel and carotenoids production in the same plant, their market price and income have been considered. The market price of biodiesel is $800/t (NESTE, 2020). So, production in the same plant, their market price and income have been calculated in $1,437.5/t without considering the glycerol credit (Ong et al., 2012). Regarding the microbial production of carotenoids, for the best scenario, the cost for microalgae derived astaxanthin was estimated in $1,830/kg, which was uncompetitive when compared to the artificial synthetic one ($1,049/kg) (Panis and Carreon, 2016). Recovering both lipids and carotenoids during the same production process, as proposed in the present study, may change this situation.

Moreover, as shown in Fig. 4, to generate 1,250 t lipids and 41 t carotenoids, 37,752 t of wheat straw are required. This means that for the global process, the yield of lipids and carotenoids can be estimated in approx. 33.1 mg/g wheat straw and 1.09 mg/g wheat straw, respectively. These numbers correspond to 0.1 g/g sugar and 3.6 mg/g sugar, respectively, taking into account the amount of sugars present in the hydrolysate. This result is similar to the production of lipids obtained by Yarrowia lipolytica from palm biomass (0.12 g/g sugar) (Intasit et al., 2020). However, the production of lipids per gram of wheat straw, as reported in the present study, could be further improved by increasing the content of sugars in the hydrolysate. Using a different pretreatment method or a combination of different pretreatment methods could help to get more sugars in the hydrolysate ( Mussatto, 2016). Regarding the carotenoid production, the yield of 3.6 mg/g sugars obtained in the present study was much more competitive than the production of 0.016 mg/g sugars reported by Rhodotorula glutinis (Elleký et al., 2019), for example. Furthermore, there is still room to improve its productivity during fermentation. Finally, improving the production and recovery of carotenoids are two potential strategies to support the industrialization of microbial biodiesel.

4. Conclusion

This study demonstrated that the production of lipids and carotenoids by R. toruloides can be maximized using similar conditions of temperature and inoculum load for fermentation. This is an important finding since it allows developing a robust bioprocess for the simultaneous production of two relevant compounds, which can facilitate the large-scale implementation. In addition, the simultaneous production of two valuable compounds, in high concentration, under similar fermentation conditions, will help reducing time, facilities, equipment and other expenses that would be required for their individual production, which at the end may significantly impact the economy of the global production process.
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