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Optimization of Carotenoids Production from *Camelina sativa* Meal Hydrolysate by *Rhodosporidium toruloides*

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Abstract: Several compounds on the market derive from petrochemical synthesis, and carotenoids are no exception. Nonetheless, since their applications in the food, feed and cosmetic sectors, and because of sustainability issues, carotenoids of natural origin are desirable. Carotenoids can be extracted from several plants but also from carotenogenic microorganisms, among which are yeasts. Nonetheless, to meet sustainability criteria, the substrate used for yeast cultivation has to be formulated from residual biomasses. For these reasons, we deploy the yeast, *Rhodosporidium toruloides*, to obtain carotenoids from *Camelina sativa* meal, an underrated lignocellulosic biomass. Its enzymatic hydrolysis ensures the release of the sugars, as well as of the other nutrients necessary to sustain the process. We therefore separately optimized enzymatic and biomass loadings, and calculated the yields and productivities of the obtained carotenoids. The best conditions (9% w/v biomass, 0.56% $w/w_{biomass}$ enzymes) were tested in different settings, in which the fermentation was performed separately or simultaneously with hydrolysis, resulting in a similar production of carotenoids. In order to collect quantitative data under controlled chemo-physical parameters, the process was implemented in stirred-tank bioreactors, obtaining 3.6 ± 0.69 mg/L of carotenoids; despite the volumetric and geometric change, the outcomes were consistent with results from the fermentation of shake flasks. Therefore, these data pave the way to evaluate a potential future industrialization of this bioprocess, considering the opportunity to optimize the use of different amounts of biomass and enzyme loading, as well as the robustness of the process in the bioreactor.

Keywords: biobased process; carotenoids; residual biomasses; *Rhodosporidium toruloides*; fermentation

1. Introduction

The widespread use of fossil resources is known to be detrimental for the environment as well as for human health [1]; therefore, alternative methods such as the development of bioprocesses based on renewable biomasses are desirable. Nonetheless, these processes are still not competitive towards the traditional petrochemical processes. Therefore, bio-based processes for commodities are undergoing a massive scientific and technological effort for improving industrial competitiveness. At the same time, in the light of the principles of cascading, an increasing effort is dedicated to the bio-based production of specialty chemicals, such as those related to the pharmaceutical, nutritional and cosmetic sectors which are requested in low amounts by the market [2–4]. Their high added value makes these molecules attractive for the scouting of bio-based processes, especially those originating from residual agro-industrial streams (often called lignocellulosic biomasses, LCBs). Such biomasses can often be valorized by the means of microbial cell factories, whose metabolism can transform carbon and energy sources of LCB origin into valuable compounds of interest [5].

In this scenario, we focused our attention on the exploitation of *Camelina* meal, the main by-product of oil extraction from *Camelina sativa* seeds [6–8]. This biomass is currently used as feed supplement; due to its macromolecular components and relatively low cost (USD 0.25/kg) [9,10], it is an attractive biomass for the development of sustainable bioprocesses. As we previously described in [11], it is possible to hydrolyse *Camelina* meal by the use of an enzymatic cocktail and provide it as the sole component of the growth medium to the yeast, *Rhodosporidium (Rhodotorula) toruloides*, for the production of carotenoids by applying separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) processes [11].

R. toruloides is naturally able to accumulate carotenoids, such as β-carotene, torulene, and torularhodin [12–14], which are produced using residual biomasses as feedstocks [15–19]. Recalling the cascading principles, carotenoids are high added value products, with a global market value expected to reach USD 2.0B by 2022 and are mainly deployed in animal feed, cosmetic, food and dietary supplements [20–23]. Since 80–90% of carotenoids on the market derive from petrochemical synthesis, there is an increasing demand for naturally produced counterparts [21].

In this work we propose process optimizations aimed at reducing the materials (enzymes and substrate loading) and fermentation time for the yeast-driven carotenoids production from *C. sativa* meal.

Enzymatic cocktails are a valuable ally in the hydrolysis of LCBs and in the release of fermentable sugars for the development of microbial-based bioprocesses [24]. Nevertheless, enzymes have costs that decisively impact second generation biorefineries, since the amount needed for the maximum hydrolysis yield is often not at an economically optimum level [25–29]. Similarly, the total solid loading is a parameter that could impact on production costs [30]; therefore, its optimization should also be considered. Furthermore, the industrial implementation of a bioprocess needs a scale-up, which may bring rise of hurdles and complications [31,32]: at the lab scale, whenever possible, we need to acquire quantitative data in a bioreactor under industrially relevant conditions.

In the present work, enzyme and biomass loadings were considered as variables to be modulated in order to release sufficient quantities of sugars for the growth of *R. toruloides* and optimize the synthesis of carotenoids. In fact, combining the best options for both enzymes and biomass loadings in a SHF process, the *R. toruloides*-productivity of carotenoids increased compared to previous data obtained from *Camelina* meal. With these data we ran fermentations at the bioreactor scale and explored the SSF and SHF to obtain quantitative data on carotenoids production by *R. toruloides* from *Camelina* meal, with the aim of promoting the industrial applications of this residual, yet underestimated, biomass.

2. Materials and Methods

2.1. Camelina Meal Hydrolysis

Flanat Research Italia S.r.l., Rho, Italy, provided *Camelina* meal derived from plants cultivated and harvested in Lombardy in 2018 and 2019. *C. sativa* seeds were processed to collect the oil, while the leftover meal was delivered to the laboratory and stored at −20 °C. Enzymatic hydrolysis of *Camelina* meal was performed using the enzyme mixture, NS22119, kindly provided by Novozymes (Novozymes A/S, Copenhagen, Denmark), as described in [11]. Without drying the biomass, different quantities of *Camelina* meal were weighted at concentrations of 3%, 6%, 9%, 12%, 15% and 20% (*w/v*) into glass bottles steeped in water with a final volume of 30 mL, and then autoclaved at 121 °C for 1 h to both sterilize and pretreat the biomass. Afterwards, enzymes were added directly to the biomass and incubated at 50 °C in a water bath under mild agitation (105 rpm). The following enzyme concentrations were tested: 11.9%, 8.93%, 5.95%, 2.98%, 2.08%, 1.04%, and 0.56% $w/w_{Camelina\ meal}$. Three independent experiments were performed.

2.2. Microbial Strain, Media and Fermentations

R. toruloides (DSM 4444) were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, GmbH, Braunschweig, Germany) and stored in cryotubes at -80 °C in 20% glycerol (v/v). The composition of the medium for the pre-inoculum was as follows (per liter): 1 g yeast extract, 1.31 g $(NH_4)_2SO_4$, 0.95 g Na_2HPO_4, 2.7 g KH_2PO_4, and 0.2 g $Mg_2SO_4 \cdot 7H_2O$. The medium was supplemented with 15 g/L of glycerol as main carbon source and a $100\times$ trace mineral stock solution consisting of (per liter): 4 g $CaCl_2 \cdot 2H_2O$, 0.55 g $FeSO_4 \cdot 7H_2O$, 0.52 g citric acid, 0.10 g $ZnSO_4 \cdot 7H_2O$, 0.076 g $MnSO_4 \cdot H_2O$, and 100 μL 18 M H_2SO_4. Yeast extract was purchased from Biolife Italia S.r.l., Milan, Italy. All other reagents were purchased from Sigma-Aldrich Co., St Louis, MO, USA.

For shake flasks fermentations, after plating on rich medium YPD (1% yeast extract, 2% peptone, 2% dextrose), a pre-inoculum with 50 mL YPD in 250 mL shake flasks was run at 30 °C and 160 rpm until stationary phase. Then, cells were inoculated at 0.2 OD at 30 °C and 160 rpm for both SHF and SSF processes (see below).

2.3. Separate Hydrolysis and Fermentation (SHF) or Simultaneous Saccharification and Fermentation (SSF)

For SHF and SSF processes, *R. toruloides* was grown in shake flasks supplemented with *Camelina* meal hydrolysate, with or without water insoluble components (WIS), which were prepared as follows. After 6 h of enzymatic hydrolysis at 50 °C, the hydrolysate was centrifuged at 4000 rpm for 10 min to separate the water-soluble components from WIS. Then, for SHF, the liquid fraction was collected and transferred into a shake flask or a stirred tank bioreactor for microbial growth at 30 °C. Alternatively, for the SSF + saccharification process, *Camelina* hydrolysate was provided directly to *R. toruloides* as growth medium, regardless of the presence of WIS. For the SSF process, *Camelina* meal was directly steeped and autoclaved in a shake flask, then supplemented with the enzymatic cocktail at 0.56% $w/w_{Camelina\ meal}$ and 0.2 OD of cells, and incubated at 30 °C and 160 rpm. These conditions were also used to calculate specific growth rate of *R. toruloides*. Three independent experiments for each setting were performed.

2.4. Batch Bioreactor Fermentation

Enzymatic hydrolysis of 1 L of *Camelina* meal 9% (w/v) was performed in 2 L shake flasks by the action of NS22119 0.56% $w/w_{Camelina\ meal}$, for 6 h at 50 °C and 130 rpm, in order to increase the homogenization of the solid component in the liquid. The hydrolysate was then centrifuged in an Avanti J-20 (Beckman Coulter Brea, CA, USA), at 4 °C and 8000 rpm for 10 min and the supernatant was collected and stored at 4 °C until use.

R. toruloides were inoculated from fresh plated cells in 250 mL flasks with 50 mL of culture medium, as a pre-seeding for the inoculation in 1 L flasks with 200 mL of the culture medium, and seed cultures were placed at 30 °C and 160 rpm for 24 h. Exponential-phase shake flasks cultures were used to inoculate bioreactors to a final optical density (OD_{600}) of 0.4. Briefly, cells were centrifuged at 6000 rpm for 10 min, washed twice with physiological solution (0.9% NaCl), and finally resuspended in 10 mL of sterilized water. The fermentations were conducted in 2 L stirred tank bioreactors (BIOSTAT® A plus, Sartorius Stedim Biotech GmbH, Goettingen, Germany) equipped with Visiferm DO ECS 225 for pO_2 measurement and Easyferm Plus K8 200 for pH measurement (both from Hamilton Bonaduz AG, Bonaduz, Switzerland). The batch fermentation was carried out with 1 L of *Camelina* meal hydrolysate, and aeration rate, agitation, and temperature were set to 1 vvm, 300 rpm (in cascade to 25% of dissolved oxygen), and 30 °C, respectively. Three independent experiments were performed.

2.5. Carotenoids Extraction

Carotenoids were analyzed by acetone extraction from *R. toruloides* cells with a protocol adapted from [33]. Briefly, 1 mL of culture broth was collected and harvested by centrifugation at 7000 rpm for 7 min at 4 °C, and the pellet was then resuspended in 1 mL

acetone and broken using glass beads by thorough agitation with a FastPrep-24™ (MP Biomedicals, LLC, Santa Ana, CA, USA). Carotenoids were extracted in the acetone phase, the suspension was centrifuged, and the supernatant collected. The extraction was repeated with fresh acetone until the biomass was colorless (four extraction rounds for each sample). Carotenoid content was measured spectrophotometrically (see below).

2.6. Analytical Methods

HPLC analyses were performed to quantify the amount of glucose, sucrose, arabinose, fructose, galacturonic acid, and acetic acid. Briefly, 1-mL samples from each of the three different streams of production (enzymatically hydrolysed *Camelina* meal, SHF or SSF) were collected and centrifuged twice (7000 rpm, 7 min, and 4 °C), and then analyzed by HPLC using a Rezex ROA-Organic Acid column (Phenomenex, Torrance, CA, USA). The eluent was 0.01 M H_2SO_4 pumped at 0.5 mL min^{-1} and column temperature was 35 °C. Separated components were detected by a refractive index detector and peaks were identified by comparing with known standards (Sigma-Aldrich).

Optical density (OD) of *R. toruloides* was measured spectrophotometrically at 600 nm. Cellular dry weight (CDW) was measured gravimetrically after drying 1 mL of cell culture (Concentrator 5301, Eppendorf AG, Hamburg, Germany).

Primary Amino Nitrogen Assay Kit (PANOPA) and Urea/Ammonia Assay Kit (K-URAMR, Megazyme International Limited, Bray, Ireland) were used to determine the amount of primary amines, ammonia and urea in the *Camelina* meal hydrolysate.

The titer of carotenoids extracted in acetone from *R. toruloides* was determined spectrophotometrically (UV-1800; Shimadzu, Kyoto, Japan) based on the maximum absorption peak for β-carotene (455 nm). A calibration curve with standard concentration of β-carotene was obtained.

2.7. Calculations and Statistical Analysis

Carotenoids yield on consumed sugars ($Y_{c/s}$) and carotenoids yield on maximum quantity of sugars per biomass ($Y_{c/b}$) measured with acid hydrolysis in [11] were calculated by Equations (1) and (2), respectively. Specific productivity (q_p) was calculated by Equation (3). Specific growth rate (μ) was calculated mathematically by an equation obtained from plotting values of OD vs. time on Excel (Figure S1). Duplication time (T_d) was calculated by Equation (4).

$$Y_{c/s} = C_p / \Delta sug \times 100 \tag{1}$$

$$Y_{c/b} = C_p / S_b \times 100 \tag{2}$$

$$q_p = C_{p/t} / CDW \tag{3}$$

$$t_d = Ln2 / \mu \tag{4}$$

where Δsug corresponded to consumed sugars, S_b to the maximum quantity of sugars in the biomass, C_p to carotenoids produced, and $C_{p/s}$ to carotenoids productivity.

For statistical analysis, a heteroscedastic two-tailed *t*-test was applied.

3. Results and Discussion

3.1. Optimization of the Enzyme Loading for Camelina Meal Hydrolysis

To release sugars from lignocellulosic biomasses, either acid or enzymatic hydrolysis is generally performed, with the latter the preferred method because of the broad enzymatic portfolio available and its mild reaction conditions, which are often more compatible with the subsequent growth of chosen microbial cell factories [24,34]. In addition, acid hydrolysis releases several inhibitory compounds that might impair microbial growth [24]. Nevertheless, glycosidic enzymes need to be evaluated as cost items; therefore, reducing the loading of enzymatic cocktails can be crucial to the economics of a bioprocess. Therefore, the yield of the sugars released must evaluate the performance of the enzymatic hydrolysis

itself and the subsequent cellular production that is of interest. In a previous work we hydrolysed 15% (w/v) of *Camelina* meal with 11.9% $w/w_{Camelina\ meal}$ of an enzymatic cocktail for the carotenoid production by *R. toruloides*, releasing sucrose, glucose, fructose, arabinose and galacturonic acid coherently and with the assessed total components and their relative abundance, as described in [11].

In this work, enzyme loadings were gradually decreased from 11.9% to 0.56% $w/w_{Camelina\ meal}$. As shown in Figure 1 (first set of bars, from the left), a significant reduction in sugar release could be measured when comparing 11.90% and 8.93% $w/w_{Camelina\ meal}$ to the lower enzymatic loadings. This could be related to the inhibitory effect of the biomass on enzymatic hydrolysis, both in terms of compounds released by the pretreatment and the sequestration by LCBs on the enzymes themselves. Nevertheless, even when starting from 5.95% and decreasing to a value of 0.56% $w/w_{Camelina\ meal}$ of enzyme loading, no significant difference could be observed, as the lowest dose also resulted in the release of approximately 15 g/L of total sugar.
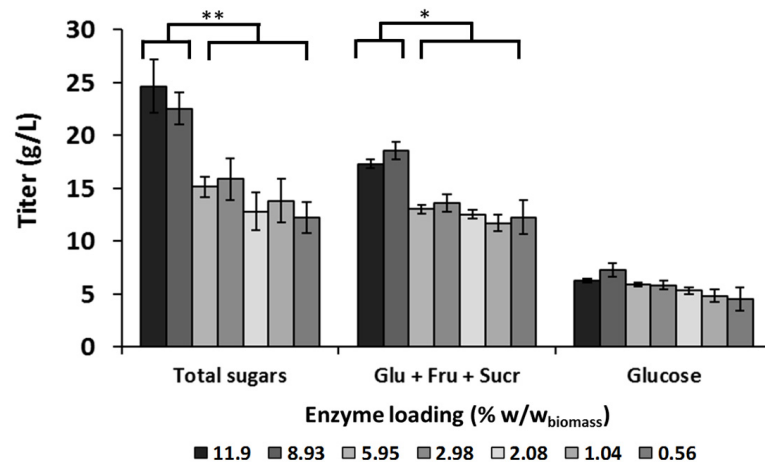

Figure 1. Sugar release from 15% (w/v) of *Camelina* meal by NS22119 enzymatic cocktail. The hydrolysis was performed at different loadings, and sugars released are shown in terms of total amount, intended as sum of sucrose, glucose, fructose, arabinose and galacturonic acid (first set of bars, from the left); the sum of sucrose, glucose and fructose (central set of bars); and sole glucose (right bars). Values are the means of three independent experiments. ** $p < 0.02$, * $p < 0.05$.

Starting from the same samples, we considered only the sugars preferentially metabolized by *R. toruloides* (Figures S2 and S3) [16,35] as a carbon and energy source (i.e., sucrose, glucose and fructose; Figure 1, central set of bars). From these data it was clear that higher amounts of enzymes mainly promoted the additional release of sugars, such as arabinose and galacturonic acid, which were not promptly consumed by *R. toruloides* when the other sugars were present (Figure S2) [11,36]. Indeed, when starting from a 5.95% $w/w_{Camelina\ meal}$ of enzyme loading, the sum of glucose, fructose, and sucrose does not significantly differ from the quantity released from the use of lower amounts of enzymes. Furthermore, if we consider only glucose, which is the first sugar to be consumed by *R. toruloides* and imposes catabolite repression on the other sugars (Figures S2 and S3), its total release from the initial biomass is similar at all of the different loadings (Figure 1, right set of bars). Therefore, a reduction in the enzymatic loading by 95% (from 11.90% to 0.56% $w/w_{Camelina\ meal}$) provided a comparable amount of glucose (as well as fructose and sucrose) in the *Camelina* meal hydrolysate, thus suggesting a possible way to reduce the cost of the overall production.

3.2. Effect of Camelina Meal Solid Loadings on the Production of Carotenoids by R. toruloides

We previously demonstrated that *R. toruloides* were able to grow and accumulate carotenoids when provided with 15% (w/v) *Camelina* meal hydrolysed with 11.9% $w/w_{biomass}$ of an enzymatic cocktail [11]. Maintaining this enzyme loading, we increased the amount

of solid loading to understand the impact on sugar release and how this would affect *R. toruloides* in a separated hydrolysis and fermentation (SHF) process.

From 20% (*w/v*) of *Camelina* meal 35.1 ± 0.07 g/L of total sugars were obtained by hydrolysis, therefore maintaining a yield (17.5 ± 0.04%) very similar to the one obtained from 15% (*w/v*) of biomass (16.4 ± 1.69%). Therefore, increasing the solid loading did not impair the enzymatic hydrolysis. Nonetheless, as shown in Figure 2A, the growth of *R. toruloides* was reduced when provided with 20% (*w/v*) *Camelina* hydrolysate compared to 15% (*w/v*), in terms of OD, and the carotenoids production significantly decreased (Figure 2B). Arguably, this could be related to the presence of the higher titers of inhibitors in the medium. This suggested that providing higher quantities of biomass was not beneficial for the obtainment of the product of interest.
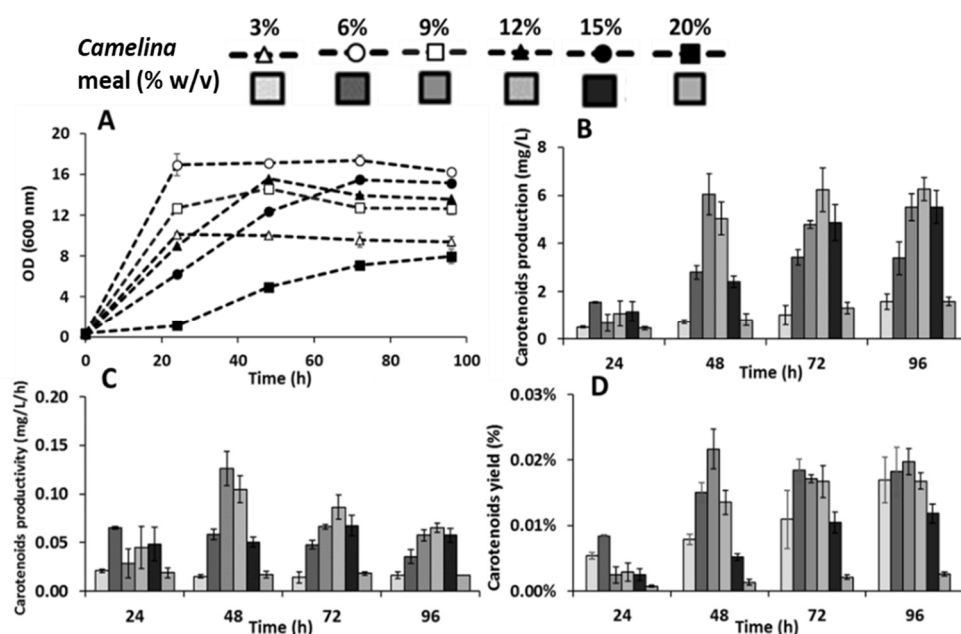

Figure 2. Production of carotenoids from different titers of *Camelina* meal. Modulation of solid loading of *Camelina* meal (from 3% to 20% *w/v*, hydrolysed with NS22119 11.9% $w/w_{biomass}$) and its effect on *R. toruloides* growth and carotenoids production. Growth in terms of OD (panel **A**), and carotenoids production in terms of titer (panel **B**), productivity (panel **C**), and yield (panel **D**) are given.

Carotenoids are produced by cells as scavenger molecules in response to environmental and cellular stresses, such as the stationary phase [11,13,37–39]. Therefore, considering the aforementioned data, we lowered the amount of biomass, and, as a result, of sugars initially provided to *R. toruloides*. In this context, cells were starved, and thus accumulated carotenoids earlier and possibly in higher amounts. The tested solid loadings were 3%, 6%, 9% and 12% (*w/v*), hydrolysed with a 11.9% $w/w_{Camelina\ meal}$ loading of the enzymatic cocktail NS22119. The derived growth media were able to support the growth of *R. toruloides*, with an anticipated entrance in the stationary phase (Figure 2A). Interestingly, the use of 6% (*w/v*) of substrate sustained a higher growth in terms of OD compared to 9% and 12% (*w/v*), most likely due to lower amounts of inhibitors in the media. Figure 2B shows the production of carotenoids over time, for each of the biomass amounts tested in the SHF process. In particular, the most interesting conditions were observed with 9% and 12% (*w/v*) of biomass, where carotenoids production obtained after 48 h of fermentation were comparable to the ones from the use of 15% (*w/v*) after 72 h of growth, which were competitive with other examples of carotenoid synthesis from residual biomasses by *R. toruloides* [15,40]. Consequently, at T48, the best productivities were reached using 9% and 12% of *Camelina* meal hydrolysate, compared to the other biomass titers tested (*p* < 0.05) (Figure 2C). Figure 2D shows that the carotenoids yield of 9% (*w/v*) after 48 h was signifi-

cantly higher compared to the yield on 12% (w/v) ($p < 0.05$), but not when compared with the yield from 6% (w/v), although this difference was at the limit of significance (most likely due to the uneven nature of the hydrolysates). Furthermore, after 48 h the productivity with 9% w/v of biomass was significantly higher compared to 6% (w/v) *Camelina* meal ($2.1 \pm 0.39 \times 10^{-5}$ h^{-1} and $0.5 \pm 0.11 \times 10^{-5}$ h^{-1}, $p < 0.02$).

Therefore, considering the solid loading of *Camelina* meal as the variable parameter of the process, the optimized conditions for carotenoids synthesis were the use of 9% (w/v) of biomass and 48 h of fermentation, with the following performances: production = 6.1 ± 0.85 mg/L, productivity = 0.13 ± 0.017 mg/L/h, specific productivity = $2.1 \pm 0.39 \times 10^{-5}$ h^{-1}, yield on CWD = $0.1 \pm 0.02\%$, yield on consumed sugars = $0.1 \pm 0.01\%$, and yield on total sugars provided = $0.02 \pm 0.003\%$. These results highlighted the importance of testing different conditions when developing bioprocesses, changing single parameters, and assessing their effect on the final product.

3.3. Combinatory Effect of Optimized Enzymes and Biomass Titers on the Production of Carotenoids by *R. toruloides*

After testing the possibility of reducing the enzymatic loading and the solid loading of *Camelina* meal, we then combined the two strategies in a single process. Therefore, we ran a SHF process in which *R. toruloides* were provided with 9% (w/v) of *Camelina* meal hydrolysed by an NS22119 enzymatic cocktail with 0.56% $w/w_{Camelina\ meal}$ as a growth medium. The results in terms of OD, sugar consumption, and carotenoids production are shown in Figure 3: samples were collected until 48 h in light of the data shown in the previous section. *R. toruloides* did not consume all the sugars provided, arguably due to the depletion of the fundamental micronutrients in the medium [11]. Furthermore, the reduction of enzymes and the consequent reduction in sugar titer contributed to the anticipation of the maximum carotenoid accumulation (2.2 ± 0.33 mg/L) at 24 h. This value was inferior compared to that obtained from the use of 11.90% $w/w_{Camelina\ meal}$ at 48 h of fermentation; nonetheless, no significant difference could be observed in terms of the productivity and specific productivity after 24 h when 0.56% $w/w_{Camelina\ meal}$ was used (0.1 ± 0.01 mg/L/h and $2 \pm 0.3 \times 10^{-5}$ h^{-1}, respectively). Although a techno-economic analysis (including downstream processing and possible related issues) has not been performed yet, it is reasonable to conclude that a reduction in both the process time and enzymatic loading would, in turn, reduce the overall cost of the process. In fact, enzyme costs are difficult to estimate: the data needed for this calculation are complex to determine because of the confidentiality surrounding some commercial enzyme productions, and also because the available costs may vary in a vast range of price, for example from USD 11.40 to USD 3.50 per kg of protein for cellulosic bioethanol production [27].
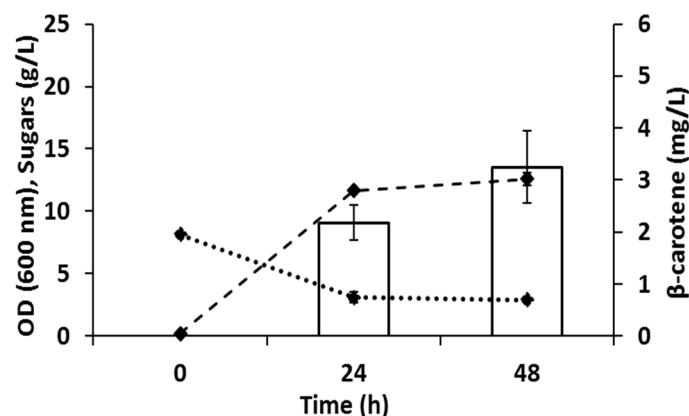

Figure 3. *R. toruloides* in SHF process with 9% (w/v) *Camelina* meal hydrolysed with 0.56% $w/w_{Camelina\ meal}$ of NS22119 as growth medium. OD (dashed line), sugar consumption (dotted line), carotenoids production (white bars). Values are the means of three independent experiments.

The optimized conditions that we identified for the synthesis of carotenoids by *R. toruloides* were the use of 9% (*w/v*) *Camelina* meal hydrolysed with 0.56% $w/w_{Camelina\ meal}$ of NS22119 as a medium for 24 h of growth in a SHF process. Furthermore, in these conditions the specific growth rate (μ) of *R. toruloides* was calculated to be 0.25 h^{-1}, with a duplication time of 2.74 h (Figure S1). In our conditions, entrance in exponential phase was calculated as reached approximately 5 h from the start of the experiment, whereas the entrance in the stationary phase after 21 h was consistent with Figure 3. Such parameters are important elements when the process is scaled-up, in order to predict the behavior of the cell factories. Since their values are dependent on the conditions of the process (e.g., microbial specie and medium), they should be calculated and evaluated singularly.

We also explored the use of the same experimental setting in SSF and SSF + presaccharification processes, to evaluate the effect of the presence of water insoluble components (WIS) as a potential stressing agent. Indeed, despite being a disturbing element for microbial growth because of uneven liquid homogenization and toxic compounds, WIS can promote the synthesis of carotenoids as scavenger molecules [11,39,41,42]. Figure 4 shows that *R. toruloides* were able to consume sugars and to produce carotenoids in both settings, reaching the maximum titer after 48 h of fermentation time (4.6 ± 0.21 mg/L for SSF + presaccharification, 4.9 ± 0.39 mg/L for SSF). The delayed production compared to SHF may be related to the harsher conditions that the cells had to face in the presence of WIS [41]. Overall, WIS do not significantly interfere with the production of carotenoids, and these fermentation conditions lead to a *Camelina* meal enriched in carotenoids, which may be directly used as a feed supplement, which is a common application in this sector, of both *Camelina* meal [9,10] and carotenoids [20,21]. This possible application permits the use of SSF to produce intracellular products such as carotenoids, that, in general, are hardly separated from the cells mixed with WIS. In addition, SSF-like processes are, generally speaking, more desirable than SHF from the industrial point of view, because of the reduction in operative steps [24]. Nevertheless, in this work we focused more on SHF due to the possibility of collecting data regarding cell growth (in terms of OD or CDW).
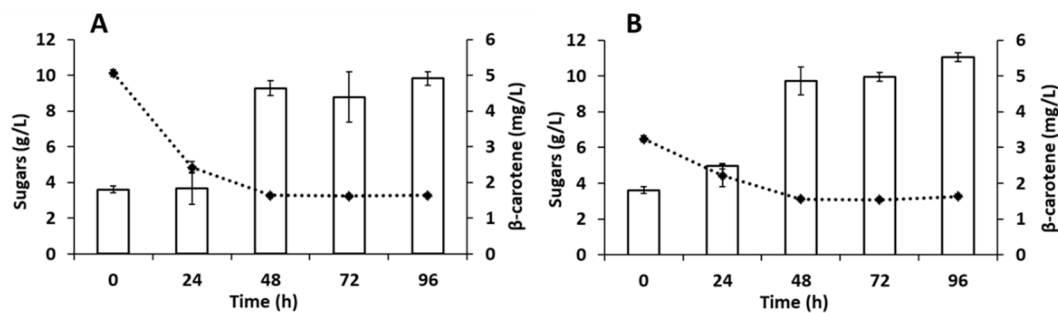

Figure 4. *R. toruloides* growth with 9% (*w/v*) *Camelina* meal hydrolysed with 0.56% $w/w_{Camelina\ meal}$ of NS22119 as growth medium in an SSF + presaccharification (panel **A**) and SSF (panel **B**) process. Sugar consumption (dotted line), and carotenoids production (white bars). OD or CDW values not available due to intrinsic turbidity of the medium in the presence of WIS. Values are the means of three independent experiments.

3.4. Carotenoids Production in Batch Bioreactors

To test the reliability of the protocols in a larger volume and to acquire data for quantitative analysis, we moved the process to stirred tank bioreactors. As a starting point, the volume of the enzymatic hydrolysis was increased to 1 L: due to the uneven nature of lignocellulosic biomasses and their inhibitory effect towards enzymatic activity, scaling up this step may have led to a decrease in the hydrolysis yield [43]. Remarkably, after 6 h of a hydrolysis of 9% (*w/v*) of *Camelina* meal by NS22119 of 0.56% $w/w_{Camelina\ meal,}$ the amount of released sugars (7.2 ± 0.84 g/L, see T0 Figure 3) was comparable with that obtained from lower volumes of hydrolysis, demonstrating the scalability of the first step of the process. Experiments in the bioreactor were performed as SHF processes, in order to collect

data regarding cellular growth, as well and to compare this with the results from shake flasks fermentations.

The obtained medium was used to test the production of carotenoids in batch bioreactors, where *R. toruloides* cells were inoculated at an initial OD of 0.4, pH 5.6 $\pm$ 0.09, and an oxygenation of 25%. Cells already reached the stationary phase after T16, as shown by the profile of OD and DCW (Figure 5A). Bioreactor cultivation permitted the monitoring of pH and dissolved oxygen: the pH increased over time to reach the value of 6.7 $\pm$ 0.03 at T48 (Figure 5B), whereas the dissolved oxygen increased after T16 (up to 80%—Figure 5A), witnessing a strong decrease in cellular growth. The increase in pH could be related to the accumulation of ammonia in the medium, initially consumed by the cells (Figure 5B), but was then produced as a consequence of amino acid catabolism triggered in response to starvation. In fact, as shown in Figure 5B, cells consumed most of the sugars and nitrogen (in the form of primary amines) at T16, although the incomplete consumption of both (which remained until the end of the fermentation) may be related to the depletion in the medium of micronutrients pivotal to cellular sustainment.
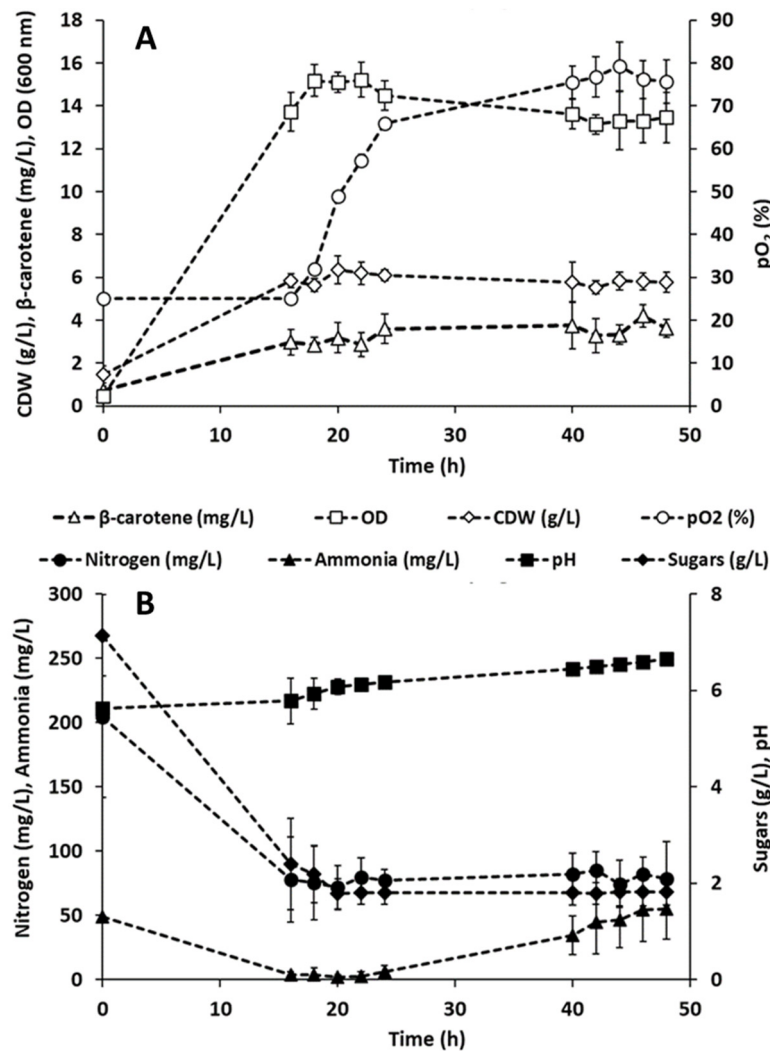

Figure 5. *R. toruloides* fermentation in stirred tank bioreactor (batch mode) with 9% (*w/v*) *Camelina* meal hydrolysed with 0.56% $w/w_{Camelina\ meal}$ of NS22119 as growth medium. Profile over time of β-carotene production (empty triangles), optical density (empty squares), cellular dry weight (empty diamonds), pO$_2$ (empty circles)—panel **A**; nitrogen titer (full circles), ammonia titer (full triangles), sugars titer (full diamonds) and pH (full squares)—panel **B**. Values are the means of three independent experiments.

The exhaustion of nutrients, the increase in pH and pO$_2$, and the growth curve profiles (in terms of OD and CDW) suggest an early entrance in the stationary phase, which is of interest for the production of secondary metabolites such as carotenoids.

Consistently, regarding the production of carotenoids, from T16 onwards there was no statistically significant increase in their accumulation, reaching 3.6 ± 0.69 mg/L after T24 (Figure 5B), with a productivity (0.13 ± 0.03 mg/L/h) comparable with the value obtained in the same conditions in shake flasks (Figure 3).

The data here disclosed are the first reports of the bioreactor scale fermentation of *Camelina* meal hydrolysate, and therefore they can pave the way for further optimization. To maximize research interests, several modifications influencing lipid and carotenoid production in yeasts, like the C/N ratio, initial CDW, pH and oxygenation [44–47], can be operated.

4. Conclusions

In the present work we demonstrate that the bioprocess involving the use of *Camelina* meal hydrolysate for the production of carotenoids by *R. toruloides* can be optimized in terms of different parameters. In fact, after dropping the enzymatic titer by 95% the hydrolysis was still efficient in releasing the main sugars consumed by the yeast in this medium (i.e., glucose, fructose, and sucrose). In parallel, we also modified the amount of total solid loading, exploring both higher and lower biomass titers than the original (15% *w/v*), in order to select the best option considering production, yield and productivity. Combining the optimized conditions for these two parameters, SHF was performed with 9% (*w/v*) *Camelina* meal hydrolysed by NS22119 0.56% $w/w_{Camelina\ meal}$, obtaining 2.2 ± 0.33 mg/L of carotenoids in 24 h, with a specific productivity of $2 ± 0.3 × 10^{-5}\ h^{-1}$. Furthermore, this SHF process in a shake flask proved to be predictable for the behavior in SSF and SSF + presaccharification, and as well when the SHF process was performed in batch bioreactors. Despite the change in both volumes and geometries, the results were consistent with those obtained from the fermentation of the shake flasks, highlighting the robustness of the developed bioprocess. The preliminary data from the bioreactor fermentation pave the way for the additional optimization of the process itself, also considering its techno-economic aspect. Based on the logic of cascading [3,48], the present work further fostered the use of *Camelina* meal as an alternative feedstock in second generation biorefineries exploiting microbial cell factories to produce fine chemicals.

E-supplementary data of this work can be found in online version of the paper.

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